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ABSTRACT

These instructor plans of instruction, lesson plans, and student text and review materials for a secondary-postsecondary-level course for medical laboratory specialist are one of a number of military-developed curriculum packages selected for adaptation to vocational instruction and curriculum development in a civilian setting. It is the first of two courses (see Note) designed to provide the necessary knowledge and techniques to perform basic procedures in a medical laboratory. The outline of instruction suggests number of hours of class time devoted to each of four subject areas, a total of 412 hours of instruction: Basic Procedures in Hematology (14 lessons, 128 hours), Basic Procedures in Immunohematology and Blood Banking (8 lessons, 64 hours), Basic Procedures in Clinical Chemistry (17 lessons, 120 hours), and Basic Procedures in Medical Bacteriology (37 lessons, 100 hours). Instructor materials include outlines of the subject areas and lesson plans listing objectives, texts, references, tools and equipment, training aids, and lecture outline. Student materials include some review materials and required excerpts from the Army Training Manual 8-227. (YLB)

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ED183828

Military Curricula for Vocational & Technical Education

MEDICAL LABORATORY SPECIALIST
(BASIC) PART I 10-14

CE 024 387

THE NATIONAL CENTER
FOR RESEARCH IN VOCATIONAL EDUCATION
THE OHIO STATE UNIVERSITY

This military technical training course has been selected and adapted by The Center for Vocational Education for "Trial Implementation of a Model System to Provide Military Curriculum Materials for Use in Vocational and Technical Education," a project sponsored by the Bureau of Occupational and Adult Education, U.S. Department of Health, Education, and Welfare.

MILITARY CURRICULUM MATERIALS

The military-developed curriculum materials in this course package were selected by the National Center for Research in Vocational Education Military Curriculum Project for dissemination to the six regional Curriculum Coordination Centers and other instructional materials agencies. The purpose of disseminating these courses was to make curriculum materials developed by the military more accessible to vocational educators in the civilian setting.

The course materials were acquired, evaluated by project staff and practitioners in the field, and prepared for dissemination. Materials which were specific to the military were deleted, copyrighted materials were either omitted or approval for their use was obtained. These course packages contain curriculum resource materials which can be adapted to support vocational instruction and curriculum development.

The National Center Mission Statement

The National Center for Research in Vocational Education's mission is to increase the ability of diverse agencies, institutions, and organizations to solve educational problems relating to individual career planning, preparation, and progression. The National Center fulfills its mission by:

- Generating knowledge through research
- Developing educational programs and products
- Evaluating individual program needs and outcomes
- Installing educational programs and products
- Operating information systems and services
- Conducting leadership development and training programs

FOR FURTHER INFORMATION ABOUT
Military Curriculum Materials
WRITE OR CALL
Program Information Office
The National Center for Research in Vocational Education
The Ohio State University
1960 Kenny Road, Columbus, Ohio 43210
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THE NATIONAL CENTER
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Military Curriculum Materials for Vocational and Technical Education

Information and Field
Services Division

The National Center for Research
in Vocational Education

Military Curriculum Materials Dissemination is . . .

an activity to increase the accessibility of military-developed curriculum materials to vocational and technical educators.

This project, funded by the U.S. Office of Education, includes the identification and acquisition of curriculum materials in print form from the Coast Guard, Air Force, Army, Marine Corps and Navy.

Access to military curriculum materials is provided through a "Joint Memorandum of Understanding" between the U.S. Office of Education and the Department of Defense.

The acquired materials are reviewed by staff and subject matter specialists, and courses deemed applicable to vocational and technical education are selected for dissemination.

The National Center for Research in Vocational Education is the U.S. Office of Education's designated representative to acquire the materials and conduct the project activities.

Project Staff:

Wesley E. Budke, Ph.D., Director
National Center Clearinghouse

Shirley A. Chase, Ph.D.
Project Director

What Materials Are Available?

One hundred twenty courses on microfiche (thirteen in paper form) and descriptions of each have been provided to the vocational Curriculum Coordination Centers and other instructional materials agencies for dissemination.

Course materials include programmed instruction, curriculum outlines, instructor guides, student workbooks and technical manuals.

The 120 courses represent the following sixteen vocational subject areas:

Agriculture
Aviation
Building & Construction Trades
Clerical Occupations
Communications
Drafting
Electronics
Engine Mechanics
Food Service
Health
Heating & Air Conditioning
Machine Shop
Management & Supervision
Meteorology & Navigation
Photography
Public Service

The number of courses and the subject areas represented will expand as additional materials with application to vocational and technical education are identified and selected for dissemination.

How Can These Materials Be Obtained?

Contact the Curriculum Coordination Center in your region for information on obtaining materials (e.g., availability and cost). They will respond to your request directly or refer you to an instructional materials agency closer to you.

CURRICULUM COORDINATION CENTERS

EAST CENTRAL
Rebecca S. Douglass
Director
100 North First Street
Springfield, IL 62777
217/782-0759

MIDWEST
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Director
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405/377-2000

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808/948-7834

MEDICAL LABORATORY SPECIALIST, PART I

Classroom Course 10-14

311-92B10

Developed by:
U.S. Army

Development and Review Dates:

May 1975

Occupational Area:
Health

Target Audiences:

Grade 11 - Adult

Print Pages: 1273
Microfiche: 22

Availability:
Vocational Curriculum
Coordination Centers

Contents:	Type of Materials:						Instructional Design:				Type of Instruction:	
	Lesson Plans:	Programmed Text:	Student Workbook:	Handouts:	Text Materials:	Audio-Visuals:	Performance Objectives:	Tests:	Review Exercises:	Additional Materials Required:	Group Instruction:	Individualized:
Annex B - Basic Procedures in Hematology	●			●	●	x	●			x	●	
Annex C - Basic Procedures in Immunohematology and Blood Banking	●			●	●		●		●	x	●	
Annex D - Basic Procedures in Clinical Chemistry	●			●	x	x	●		●	x	●	
Annex E - Basic Procedures in Medical Bacteriology	●			●	●	x	●			x	●	

X Materials are recommended but not provided.

THE NATIONAL CENTER
FOR RESEARCH IN VOCATIONAL EDUCATION

OSU
The Ohio State University

1960 Kenny Road
Columbus, Ohio 432
(614) 486-3655

Course Description:

This course was designed to provide the necessary knowledge and techniques to perform basic procedures in a medical laboratory. The course consists of four subject areas covering 412 hours of instruction. Annex A was deleted due to the military specific material contained.

Annex B - Basic Procedures in Hematology consists of 14 lessons covering 128 hours of instruction.
- Equipment (1 lesson, 1 hour)
- Venipuncture (1 lesson, 2 hours)
- Blood Analysis (6 lessons, 14 hours)
- Blood Coagulation (2 lessons, 2 hours)
- Hematological Techniques (2 lessons, 75 hours)
- Written and Practical Exams (2 lessons, 34 hours)

Annex C - Basic Procedures in Immunohematology and Blood Banking contains 8 lessons requiring 64 hours of instruction.
- ABO, Rh, Du Confirmation (2 lessons, 3 hours)
- Antihuman Globulin Tests (1 lesson, 1 hour)
- Blood Donor Compatibility (2 lessons, 4 hours)
- Immunohematological Techniques (1 lesson, 40 hours)
- Exams (2 lessons, 16 hours)

Annex D - Basic Procedures in Clinical Chemistry consists of 17 lessons requiring 120 hours of instruction.
- Introduction (1 lesson, 3 hours)
- Mathematics (1 lesson, 12 hours)
- Spectrophotometry (1 lesson, 3 hours)
- Volumetric Glassware and Quality Control (2 lessons, 3 hours)
- Various Chemical Tests (7 lessons, 71 hours)
- Laboratory Instrumentation (1 lesson, 4 hours)
- Exams (4 lessons, 24 hours)

Annex E - Basic Procedures in Medical Bacteriology contains 37 lessons covering 100 hours of instruction.
- Introduction (1 lesson, 1 hour)
- General Laboratory Techniques (3 lessons, 9 hours)
- Bacteria Cultures (6 lessons, 8 hours)
- Gram Stain (4 lessons, 9 hours)
- Cocci Biochemical Tests (3 lessons, 7 hours)
- Throat Specimen (2 lessons, 5 hours)
- Sterilization and Decontamination (2 lessons, 6 hours)
- Enteric Biochemicals (3 lessons, 4 hours)
- Intestinal Pathogens (2 lessons, 7 hours)
- Kirby Bauer Test (2 lessons, 3 hours)
- Identification Schemes (2 lessons, 13 hours)
- Cerebrospinal Fluid (2 lessons, 7 hours)
- Exams (5 lessons, 20 hours)

The course contains both teacher and student materials. The printed instructor materials include annex outlines and lesson plans listing objectives, texts, references, tools and equipment, training aids, and lecture outline. Student materials include some review materials and excerpts from Army Training Manual 8-227, Sections 3, 4, 5.

MEDICAL LABORATORY SPECIALIST (BASIC) COURSE

PART I

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Reference: Training Manual 8-227-5	1256

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SECTION I - PREFACE

A. Course: 311-92B10, Medical Laboratory Specialist (Basic).

B. Purpose: To provide graduates of this course with the necessary knowledge and techniques to perform basic procedures in a medical laboratory. MOS for which trained: 92B10.

C. Instructional objectives:

*1. Given prepared specimens, consistently perform each of four fundamental hematological procedures of the complete blood count with an acceptable degree of accuracy when compared with the established value of each specimen.

*2. Given prepared specimens, consistently perform each of the fundamental blood banking procedures, to include ABO groups, Rh types, antiglobulin tests, and compatibility testing with an acceptable degree of accuracy when compared to the established factors in each specimen.

*3. Given a series of prepared specimens of simulated body fluids having constituent concentrations that represent clinical values, test each specimen utilizing the chemistry procedure specified to attain satisfactory values to include mathematical calculations of solution preparation and related problems.

*4. Given necessary materials and bacterial cultures, perform interpret, and report gram stains, isolation streaking, morphological characteristics, biochemical tests, and identification of bacteria. Prepare bacteriological media, and discuss and perform sterilization/ decontamination procedures.

2

SECTION I - PREFACE (Cont)

D. Prerequisites: Enlisted grade E4 or below. Credit for high school level courses in chemistry and algebra. Normal color vision (using standard color discrimination plates, not missing more than three plates). No adversion to sight of blood or needles.

MODIFICATIONS

Section II - Summary of this publication has (have) been deleted in adapting this material for use in Vocational and Technical Education. Deleted material involves extensive use of military forms, procedures, systems, etc. and was not considered appropriate for use in vocational and technical education.

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SECTION III - BODY

ACADEMIC SUBJECTS

MEDICAL LABORATORY PROCEDURES (BASIC) COURSE

Division and Subject or Annex Title & Subject	Annex
Laboratory Science Division	
Microscope and Laboratory Equipment	B
Venipuncture	B
Blood Dilutions and Cell Enumeration	B
Capillary Puncture and Hemoglobin Determinations	B
Leukocyte Identification and Staining	B
Hematocrit and Sedimentation Rate	B
Introduction to Blood Coagulation	B
Bleeding Time and Coagulation Time	B
Thrombocyte Count and Prothrombin Time	B
Erythrocyte Morphology	B
Miscellaneous Hematological Procedures	B
Hematological Techniques	B
Written Examination and Critique I	B
Practical Examination and Critique I	B
ABO Grouping and Confirmation Tests	C
Rh Typing and Du Determination	C
Antihuman Globulin Test	C
Blood Donor Processing	C
Compatability Testing	C
Immunohematological Techniques	C
Written Examination and Critique II	C
Practical Examination and Critique II	C
Introduction to Clinical Chemistry	D
Mathematics and Solutions	D
Spectrophotometry	D
Specimen Processing & Volumetric Glassware	D
Quality Control	D
Carbohydrates	D
Protein and Kidney Function	D
Chloride	D
Practical Examination & Critique III	D
Practical Examination & Critique IV	D
pH and Blood Gases	D
Sodium and Potassium	D
Bilirubin	D
Enzymes	D
Laboratory Instrumentation	D

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SECTION III - BODY (Cont)

Division and Subject or Annex Title & Subject	Annex
Written Examination and Critique V	D
Practical Examination and Critique V	D
Introduction to Bacteriology	E
Sterilization and Decontamination	E
Media Preparation	E
Media Preparation Practical Exercise	E
Nutrition and Cultivation of Bacteria	E
Streaking, Selection and Morphology of Isolates	E
Streaking, Selection and Morphology of Isolates Practical Application	E
Gram Stain Procedures	E
Gram Stain Practical Exercise	E
Biochemical Testing Procedures	E
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Staphylococcus Species	E
Neisseria Species	E
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Enteric Biochemicals	E
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Station to Station Examination	E
Miscellaneous Bacteria of Medical Importance	E
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Introduction to Identification Schema	E
Mixed Organisms - Practical Exercise	E
Collecting and Handling of Clinical Specimens	E
Media Preparation Examination	E
Identification of Bacteria - Practical Examination	E
Darkfield Microscopy	E
Written Examination	E

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MODIFICATIONS

Annex A-Orientation of this publication has (have) been deleted in adapting this material for use in Vocational and Technical Education. Deleted material involves extensive use of military forms, procedures, systems, etc. and was not considered appropriate for use in vocational and technical education.

SECTION IV - ANNEXES

ANNEX B

BASIC PROCEDURES IN HEMATOLOGY

OBJECTIVE: Given prepared specimens, consistently perform each of four fundamental hematological procedures of the complete blood count with an acceptable degree of accuracy when compared with the established value of each specimen.

Subject and LP No	Lesson Objective	References
Microscope and Laboratory Equipment 08-280-006	List the four main systems of the laboratory microscope and their component parts. Briefly discuss the procedures involved in using the microscope. Briefly discuss the routine maintenance procedures for the microscope. List and briefly discuss the general characteristics of the laboratory centrifuge and the precautions for its use. List the parts of the spectrophotometer and their function.	TM 8-227-4, pp 1-10 thru 2-3.
Venipuncture 08-280-007	List and discuss the steps in blood collection technique by venipuncture using both syringe and vacutainer equipment. List and discuss the anticoagulants used for collection and preservation of blood specimens.	TM 8-227-4, pp 3-1 thru 3-6; VT 10.
Blood Dilutions and Cell Enumeration 08-280-008	List and discuss the proper steps for performing blood dilutions and cell enumerations, to include calculations, units of report, and normal values.	TM 8-227-4, pp 5-1 thru 5-10.
Capillary Puncture and Hemoglobin Determination 08-280-011	List and explain the proper steps in performing a capillary puncture. Name five forms and three genetic types of hemoglobin. Classify the four hemoglobin determinations discussed during this lecture as gaavimetric or colorimetric, and direct or	TM 8-227-4, pp 3-6 thru 3-8, 4-11 thru 4-18; VT 11.

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Subject and LP No	Lesson Objective	References
	indirect, and as visual or photometric methods. List and explain the proper steps in performing a hemoglobin determination by the cyanmethemoglobin technique. State the normal value of hemoglobin concentration for adults.	
Leukocyte Identification and Staining 08-280-009	State the characteristics of a thin blood smear suitable for microscopic examination. Explain the procedure for performing the Wright's staining technique and name the components of the stain and the sources of error encountered. Outline the procedure for preparing and staining a thin blood smear for microscopic examination. Identify the various normal leukocytes and list their characteristics. State the criteria used in their identification. Discuss the purposes and principles of the differential count and cite both normal and abnormal conditions encountered.	TM 8-227-4, pp 5-1 thru 5-10 ; VT 803.
Hematocrit and Sedimentation Rate 08-280-014	Briefly describe the principles on which hematocrit and erythrocyte sedimentation rate are based and explain common sources of error encountered. List the normal value ranges and proper units of report for microhematocrit and Wintrobe sedimentation rates. List and describe the equipment necessary to perform erythrocyte sedimentation rates and microhematocrit determinations. List in order the steps in the performance of microhematocrit and erythrocyte sedimentation rates.	TM 8-227-4, pp 4-20 thru 4-26.

Subject and LP No	Lesson Objective	References
Introduction to Blood Coagulation 08-280-045	Explain the coagulation theory. List the phases of coagulation in order.	Miale, chap 14, pp 999-1074.
Bleeding Time and Coagulation Time 08-280-017	Explain and discuss the principles and procedures used in determining the bleeding time and coagulation time. List the normal range and sources of error for each determination. List the normal range of these determinations and correlate abnormal results with specific coagulation defects. Perform these two procedures and report the results with acceptable accuracy.	TM 8-227-4, pp 4-1 thru 4-5.
Thrombocyte Count and Prothrombin Time 08-280-018	Explain the origin and list the functions of platelets. List the functions of prothrombin and explain the purpose of performing the prothrombin time. List the steps in the procedures and sources of error in performing the direct platelet count, platelet estimate, and prothrombin time. List the correct units of report and normal values.	TM 8-227-4, pp 6-7 thru 6-12, 6-16 thru 6-17; VT 318.
Erythrocyte Morphology 08-280-025	State the origin and stages of maturation of the erythrocyte. State the characteristics and descriptive terminology used to identify and classify each of the normal and abnormal forms of the erythrocyte. After practice, evaluate and report erythrocyte morphology.	TM 8-227-4, pp 4-1 thru 4-5.

Subject and LP No	Lesson Objective	References
Miscellaneous Hematological Procedures 08-280-027	**List and explain the steps in performing the reticulocyte count and interpret the results of testing. Outline the procedure for performing cerebrospinal fluid examinations, with emphasis on normal values and calculations. Discuss the two forms of sickle cell disease, with emphasis on severity and laboratory findings. Outline the sickle cell preparation procedure and explain the results of testing.**	**TM 8-227-4, pp 4-10 thru 4-11, 4-18 thru 4-19, pp 5-14 thru 5-15.**
Hematological Techniques 08-280-013	**Carefully select and prepare specimens, reagents, controls, and carry out proper utilization of procedures and materials. Properly perform tests and procedures, interpret results, resolve discrepancies, and accurately record and classify results.**	**All previous references.**
Written Examination and Critique I **08-280-034**	**Answers questions on theories, principles, and procedures presented in lecture and reading assignments.** Demonstrate an ability **to calculate and interpret the results of hematological test procedures given specific and example test data.**	**All previous references.**
Practical Examination and Critique I **08-280-035**	**Demonstrate** dexterity utilizing various equip**ment (microscope, pipets, slides, hemacytometer, lancets,** and mechanical **and automated devices) with a degree of accuracy equivalent to at least 70 percent.**	**All previous references.**

BIBLIOGRAPHY

Miale, John B. Laboratory Medicine: Hematology. 3d ed. St. Louis: C. V. Mosby Company, 1967.

SUPPLEMENTAL BIBLIOGRAPHY

Diggs, L. W., and others. The Morphology of Blood Cells. North Chicago: Abbott Laboratories, 1954.
Gradwohl, R. B. H., and others. Clinical Laboratory Methods and Diagnosis. St. Louis: C. V. Mosby Company, 1963.
Seiverd, C. E. Hematology for Medical Technologists. Philadelphia: Lea and Febiger, 1964.
Wintrobe, M. M. Clinical Hematology. 3d ed. Philadelphia: Lea and Febiger, 1967.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-000-1
LABORATORY SCIENCES DIVISION 018
CLINICAL MICROSCOPY BRANCH

Hematology Lecture Objectives

The following is a list of hematology objectives, outlined per lecture as indicated, for the 311-92B10, Medical Laboratory Specialist Course (Basic).

A. LP 08-280-006, Microscope

1. List the four main microscopic systems and their component parts.

2. List the necessary procedures involved in the proper use of the microscope.

3. List the routine maintenance procedures for the microscope.

B. LP 08-280-007, Venipuncture

*1. Given the proper equipment, perform a venipuncture by the syringe and vacutainer method as presented in class.

2. List the anticoagulants used for collection and preservation of blood specimens, and discuss their proper usage as presented in class.

C. LP 08-280-008, Blood Dilutions and Cell Enumerations

1. List in order the proper steps for performing both red blood cell and white blood cell dilutions and enumerations.

2. List the units of reports, and normal values for both the red blood cell and white blood cell enumerations.

*3. Given the necessary data, calculate and report the red blood cell and white blood cell count to the nearest whole number utilizing the correct unit of report.

D. LP 08-280-011, Capillary Puncture and Hemoglobin Determination

1. Given proper equipment, perform a capillary puncture using the procedure stated in class.

*2. Perform a hemoglobin determination by the cyanmethemoglobin method using blood obtain from both venipuncture and capillary puncture.

3. List all proper steps in using the spectrophotometer for the cyanmethemoglobin determination and state improper procedure.

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* This mimeo supersedes M 08-280-000-1, 076.

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E. LP 08-280-009, Leukocyte Identification and Staining

1. Identify the steps in the procedure and sources of error for performing the leukocyte differential.

2. Identify the criteria employed for the identification of leukocytes.

3. Identify the criteria used to classify the various types of normal and abnormal leukocytes and the pathological conditions associated with the increase of each type.

F. LP 08-280-014, Hematocrit and Sedimentation Rate

1. State the proper steps for performing microhematocrit and sedimentation rates.

2. Given sources of error select the manner in which a hematocrit or sedimentation rate result would be affected.

3. Select the normal values and proper units of report for microhematocrits and Wintrobe sedimentation rates.

G. LP 08-280-045, Introduction to Blood Coagulation

1. List the three systems which are involved in hemostasis.

2. List the three stages of coagulation in proper sequence.

H. LP 08-280-017, Bleeding Time and Coagulation Time

1. Identify the two methods for determining bleeding time.

2. Identify the one method for determining coagulation time.

3. List the normal range for bleeding times and coagulation time.

I. LP 08-280-018, Thrombocyte Count and Prothrombin Time

1. State the origin and list the functions of platelets.

2. List the functions of prothrombin and the purpose of performing the prothrombin time.

3. List the steps in the procedures and the sources of error in performing the direct platelet count, platelet estimate, and prothrombin time.

    4. List the correct units of report and normal values.

J. LP 08-280-025, Erythrocyte Morphology

    1. Identify the stages of maturation of the erythrocyte.

    2. Be able to recognize the characteristics and descriptive terminology used to identify and classify each of the normal and abnormal forms of the erythrocyte.

K. LP 08-280-027, Miscellaneous Hematological Procedures

    1. Select the procedural steps involved in performing the reticulocyte count, to include principle, reagents, sources of error and significant findings.

    2. Select the procedure for performing a cerebrospinal fluid examination, with emphasis on reagents, calculations and normal values.

    3. List the two forms of sickle cell anemia, and select the laboratory findings for both.

L. LP 08-280-013, Hematological Techniques

    1. Carefully select and prepare specimens, reagents, controls and carry out proper utilization of procedures and materials.

    2. Properly perform tests and procedures, interpret results, resolve discrepancies and accurately record and classify results.

M. LP 08-280-034, Written Examination and Critique I

    1. Answer questions on theories, principles and procedures presented in lecture and reading assignments.

    2. Demonstrate an ability to calculate and interpret the results of hematological test procedures given specific and example test data.

N. LP 08-280-035, Practical Examination and Critique I

    1. Given prepared specimens, consistently perform WBC counts, hemoglobins and hematocrits with a degree of accuracy equivalent to established guidelines.

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M 08-280-000-1
018

2. Given prepared specimens, consistently perform differential cell counts with a degree of accuracy equivalent to established guidelines.

3. Report results of the above mentioned hematological examinations in correct format, using proper units of report.

4. Perform a venipuncture utilizing acceptable technique as prescribed.

---

*terminal objective.

SP5 Paul N. Steinike
*LP 08-280-006
048

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
CLINICAL MICROSCOPY BRANCH

Microscope (1 hour)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic)

PLACE: Classroom

REFERENCES: TM 8-227-4
TM 8-227-6

STUDY ASSIGNMENT: After class study TM 8-227-4 pp 2-6 thru 2-12.

STUDENT UNIFORM AND EQUIPMENT: Not Applicable

TOOLS, EQUIPMENT, AND MATERIALS: None

PERSONNEL: One primary instructor (MOS 92B4H, or 92B3H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, eraser, overhead projector and transparencies.

TROOP REQUIREMENTS: None

TRANSPORTATION: None

METHOD OF INSTRUCTION: Lecture

I. INTRODUCTION (5 min)

A. Opening Statement: For the clinical technician to perform valid hematological tests he must have a working knowledge of the microscope. Not only will this knowledge allow the technician to perform valid, precise testing, but it will help to prolong the service life of the microscope.

B. Objectives

1. List the four main microscope systems and their component parts.

2. Briefly describe the necessary procedures involved in the use of the microscope.

3. List the routine maintenance procedures for the microscope.

C. Class Procedure and Lesson Tie-in

1. Practical exercises utilizing the microscope will follow the lecture.

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*This lesson plan supersedes LP 08-280-006, 075

2. If necessary individual conferences will be held to solve individual problems.

II. **EXPLANATION** (38 min)

A. Microscope systems

1. Framework system

a. Base

(1) Instrument foundation
(2) Component housing

(a) Mirror
(b) Substage lamp

b. Arm - connecting support
c. Body - contains some optics
d. Stage

(1) Fixed stage
(2) Mechanical stage

e. Revolving nose piece - houses objectives

2. Illumination system

a. Light sources

(1) Internal or built-in source
(2) External sources

(a) Sunlight
(b) Special external lamps

b. Mirror

(1) Used with all external light sources
(2) Has two sides

(a) Planar side - for artificial light
(b) Concave side - for sunlight

QUESTION: Why is a concave mirror used when sunlight is used as a light source?

ANSWER: Sunlight is weaker and more diffuse than an artificial light; therefore, a concave mirror is used in order to concentrate the sunlight.

c. Abbe condenser

  (1) Consists of a series of lens
  (2) Further intensifies the light
  (3) Condenser positioning

    (a) High position - High intensity light
    (b) Low position - Low intensity light

d. Iris diaphragm

  (1) System of small shutters
  (2) Controls glare

3. Magnification system

  a. Ocular lens or eyepiece - 10x power
  b. Objective lenses

    (1) Normally three

      (a) Low power: 10x
      (b) High power: 43x-47x
      (c) Oil immersion: 95x-100x

    (2) Are par focal

QUESTION: What is meant by the term par focal?

ANSWER: The technician can switch from one objective to another without extreme changes in focus.

  c. Total magnification = ocular magnification x objective magnification

4. Focusing system

  a. Coarse adjustment

    (1) Large inner knob
    (2) Finds desired plane of focus

  b. Fine adjustment

    (1) Smaller outer knob
    (2) Sharpens and defines image

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B. Use of the Microscope

1. Place the low power objective in position.
2. With the coarse adjustment place the nosepiece in the down position.
3. Lower the Abbe condenser to the down position.
4. Using the coarse adjustment focus upward.
5. Use the fine adjustment to sharpen image.
6. Switch to a lighter objective.
7. Readjust the Abbe condenser.
8. Refocus image, using the fine adjustment.
9. For oil immersion objective add oil between changing of objectives.

C. Microscope maintenance

1. Routinely performed by the technician
2. Storage
    a. In a warm, dry, dust-free environment
    b. Kept covered when not in use

3. Care of lenses

    a. Cleaning

        (1) Use only lens paper
        (2) Xylene - only for difficult, cleaning problems

    b. Immersion oil

        (1) Removed immediately after use
        (2) Prevention of build-up

    c. Storage - damage prevention

        (1) Low power objective in position
        (2) Abbe condenser fully raised

III. SUMMARY (2 min)

A. Review of Main Points

1. Microscope systems
2. Use of Microscope
3. Microscope maintenance

B. Closing Statement: The microscope is an invaluable tool for the medical laboratory specialist. A working knowledge of its structure and use gives the user distinct advantages in his work. Not only will he perform valid tests, but with routine maintenance he will decrease down time for repairs and prolong the service life of the microscope.

SP5 Paul N. Steinike
*IM 08-280-006
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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
CLINICAL MICROSCOPY BRANCH

Microscope

For the clinical technician to perform valid hematological tests he must have a working knowledge of the microscope. Not only will this knowledge allow the technician to perform valid, precise testing, but it will help to prolong the service life of the microscope.

Although styles and models of microscopes vary, they all contain basically the same four systems: the framework, illumination, magnification, and focusing systems.

The first of these, the framework system, contains those elements of the microscope which have as their function structural support. The base forms the foundation for the microscope. It also serves as a housing for some components such as the mirror or substage lamp. The arm serves as a connecting support between the base and the body. At the upper end of the arm is the body or body tube. The body tube supports part of the microscopes optics. The platform located on the lower portion of the arm is the stage. The slide rests on the stage which may be either a fixed stage or mechanical stage. The fixed stage is a rigid platform and the slide must be moved by hand when scanning material on the slide. The mechanical stage has a series of screws which allows the technician to move the slide mechanically, thus increasing accuracy when performing a systematic scan. The revolving nosepiece is the final component of the framework system. It is attached to the body directly over the stage, and is the component which houses the objectives.

The second microscope system is the illumination system. The most important component of this system is the light source. Light sources may be of two general types: internal or built-in light sources, and external light sources. Built-in sources consist of a small substage lamp that is housed in the microscopes base. External light sources include sunlight (rarely used) and specially designed microscope lamps. When an external light source is used a mirror must be employed. The mirror is attached to the base and has two sides, one planar (flat) and the other concave. (curv[illegible]. The planar side is used in conjunction with an external lamp. Since sunlight tends to be more diffuse, the concave mirror must be employed to further concentrate the light.

No matter what the light source, the light must be further concentrated in order to achieve optimum effect. To achieve this, the microscope is equipped with an Abbe condenser. The condenser is an encased series of

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*This Instructor's Manuscript supersedes IM 08-280-006, 075

lens mounted on a track below the stage. The ability to control the position of the condenser further adds to light intensity control. If the condenser is at its highest point the light intensity is highest; when fully lowered the light intensity is at its lowest. Since light intensity must be increased, the Abbe condenser becomes an indispensible component of microscopes with interchangeable lenses.

Normally at the bottom of the Abbe condenser is the iris diaphragm. This is a system of shutters controlled by an attached lever. The function of the iris diaphragm is to control glare from the condenser.

The heart of the microscope is the magnification system. Essentially this system consists of two small microscopes fused into one, thus giving rise to the term "compound microscope." The first of these minor microscopes is the ocular lense or eyepiece, which has a magnification of 10x. The objective lenses make up the second minor microscope; they form the initial magnified image. Most laboratory microscopes carry three objective lenses: a green-banded low power lens with 10x magnification; a yellow-banded high power lens with a magnification between 43x-47x; and a red-banded oil imminsion lens that magnifies between 95x-100x. The objective lens are par focal which allows the technician to switch from one objective to another without extreme changes in focus.

With the compound microscope total magnification is calculated by multiplying the ocular magnification by the objective magnification. For example, utilizing a 10x ocular and high power objective the total magnification would be between 430x to 470x.

The fourth and last system of the microscope is the focusing system. This is composed of two components: the coarse and fine adjustment knobs. The coarse and fine adjustments are located just above the base on the arm. The coarse adjustment knob is the large inner one, which is used to bring the microscope into the desired plane of focus. The smaller outer knob makes the fine adjustments; it is employed to obtain a sharp, well-defined image.

The procedure for using a microscope varies depending upon the type of examination being performed and the skill level of the technician. The following procedure is the most commonly used and is the one used here. First, place the slide right side up on the stage, turn on the illuminator and switch to the low power objective. Utilizing the coarse adjustment, lower the objective to its lowest setting. Since low power does not require a high intensity light, also lower the Abbe condenser to its lowest point. Now using the coarse adjustment and focusing upward bring the object into focus. By always focusing upward the objective lens will not ram into the slide and cause damage to either. The fine adjustment is now used to define and sharpen the image. If a higher magnification is desired, the technician may then switch to a higher objective. The Abbe condenser is adjusted as necessary and the fine adjustment used to correct any minor changes in focus.

The procedure is slightly varied when the oil immersion objective is to be employed. Before switching the oil immersion lens into place, a drop of oil is placed on the slide directly below the spot where the objective will come to rest. The oil immersion objective is then lowered into place and the rest of the procedure carried out.

Maintenance of the laboratory microscope is of upmost importance. The technician is responsible for performing routine maintenance and insuring that the microscope is properly stored. Proper storage is a necessity if deterioration of the microscope is to be prevented. Storage should be in a warm, dry, dust-free environment. As an added precaution always keep the microscope covered when it is not in use. When properly cared for the microscope will render long, dependable service.

Proper maintenance of the microscope ocular lens and objectives cannot be overemphasized. The cardinal rule of lens care is to never touch the l[illegible] with anything but lens paper. Occasionally difficult cleaning problems will arise and it will be necessary to use a very small amount of xylene on the lens paper. When immersion oil is employed it should be removed immediatley after use; this will prevent residue buildup which can effect the optical properties of the lens. When the microscope is not in use, the low power objective should be in position and the Abbe condenser fully raised. This will prevent damage to the high power objectives and also protect the light source or mirror should the instrument be jarred.

The microscope is an invaluable tool for the medical laboratory specialist. A working knowledge of its structure and use gives the user distinct advantages in his work. Not only will he perform valid tests, but with routine maintenance he will decrease down time for repairs and prolong the service life of the microscope.

SSG Gerald Fayette

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-007
LABORATORY SCIENCES DIVISION 046
CLINICAL MICROSCOPY BRANCH

Venipuncture (2 hours)

COURSES PRESENTED TO: 311-92B20, Medical Laboratory Procedures (Basic)

PLACE: Laboratory

REFERENCES:

TM 8-227-4

STUDY ASSIGNMENT: Study TM 8-227-4 p 3-1 to 3-5 after class.

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: None

PERSONNEL: One primary instructor (MOS 92B3H or 92B4H) and two secondary instructors (MOS 92B3H or 92B4H)

INSTRUCTIONAL AIDS: Chlak, chalkboard, and eraser, television receivers and VT No. 10.

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

FIRST HOUR

I. INTRODUCTION (10 min)

A. Opening Statement: In the 17th century, the artist Christopher Wren and a chemist, Robert Boyle, performed the first procedure for entry into a living blood vessel. Their procedure utilized a quill attached to a primitive syringe. When Boyle and Wren operated on a dog, the quill was inserted through a lesion made in a blood vessel by a lancet. During this exercise, you will

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*This lesson plan supersedes LP 08-280-007, 014.

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learn all the precautions and techniques for performing the modern equivalent of this venipuncture, as well as the various anticoagulants used in this and other tests. It is a delicate surgical procedure, requiring the technician's fullest attention and skill.

B. Objectives

1. List and discuss the steps in blood collection technique by venipuncture, using both syringe and Vacutainer equipment.

2. List and discuss the anticoagulants used for collection and preservation of blood specimens.

C. Class Procedure and Lesson Tie-in

1. Venipuncture by syringe will be demonstrated by Videotape #10 immediately following the lecture. Students will perform a venipuncture utilizing a syringe after viewing the videotape.

2. Venipuncture by Vacutainer method will be reviewed and performed at a later date.

II. EXPLANATION (55 min)

A. Principle

B. Techniques

1. Syringe method

NOTE: Show pieces of equipment as they are discussed.

a. Equipment

QUESTION: What obvious pieces of equipment are needed for a syringe venipuncture?

ANSWER: A syringe and a needle.

(1) Syringe (normally 10 cc)

(2) Needle (19-23 gauge)

QUESTION: Is a 19 gauge needle smaller or larger than a 21 gauge?

ANSWER: Larger.

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(3) Collection tube

(4) Tourniquet

(5) Alcohol (isopropyl or ethyl), 70 percent

(6) Gauze or cotton balls, 2" x 2"

b. Procedure

(1) Preparation for venipuncture

(a) Assemble equipment

(b) Stand while performing venipuncture

(2) Selection of site for venipuncture

(a) Place tourniquet (quick release knot) on upper arm (elastic band with Velcro fastener often used)

(b) Palpate and view (area of choice: antecubital fossa)

(c) Note vein orientation

(d) Release tourniquet

(3) Cleanse puncture site using spiral or parallel, non-over-lapping strokes

NOTE: Insure students perform a second cleansing if the first is inadequate.

(4) Puncture

(a) Replace tourniquet

(b) Anchor vein

QUESTION: Why is it necessary to anchor the vein?

ANSWER: To stabilize the vein, since some veins roll aside at entry.

(c) Puncture at 30° angle with bevel up

(d) Thread vein

(e) Aspirate blood by slowly retracting plunger (normally 7 cc)

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NOTE: Emphasize the importance of not switching hands at this point.

- (f) Release tourniquet (2 min maximum)
- (g) Remove needle; apply gauze

(5) Post-puncture care

- (a) Observe patient
- (b) Carefully transfer blood to collecting tube, mix

SECOND HOUR

2. Vacutainer method

   a. Equipment

   - (1) Vacutainer holder (re-usable)
   - (2) Needle (20 gauge)
   - (3) Collection tube
   - (4) Tourniquet
   - (5) Alcohol (isopropyl or ethyl), 70 percent
   - (6) Gauze or cotton

   b. Procedure

   (1) Preparation for venipuncture

   - (a) Assemble equipment
   - (b) Stand while performing venipuncture

   (2) Selection of site for venipuncture

   - (a) Place tourniquet on upper arm
   - (b) Palpate and view: (area of choice: antecubital fossa)
   - (c) Note vein orientation
   - (d) Release tourniquet

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(3) Cleanse puncture site

(4) Puncture

  (a) Replace tourniquet

  (b) Anchor vein

  (c) Puncture at 30° angle

  (d) Thread vein

  (e) Aspirate blood by pushing tube onto needle (automatic fill)

  (f) Release tourniquet (2 min maximum)

  (g) Remove needle, apply gauze

(5) Post-puncture care

  (a) Observe patient

QUESTION: One of the most frequently occurring reactions to a venipuncture is syncope (fainting). What first aid is indicated in this situation?

ANSWER: Remove needle; lay patient down with feet elevated; loosen clothing; revive with ammonia; remove patient from view of other patients.

QUESTION: Another reaction to a venipuncture is a hematoma, or sub-surface bleeding at the site of puncture. What procedure should be followed if this occurs during the venipuncture?

ANSWER: Remove the needle, apply gauze with pressure for several minutes.

  (b) Remove tube and mix

3. Sources of error

  a. Technician's difficulties

    (1) Nervousness

    (2) Handling equipment

  b. Patient's difficulties

    (1) Behavior

    (2) Physiologic condition of veins, tissue

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C. Anticoagulants

  1. Definition

    a. Prevents clotting

    b. Results in cells and plasma

  2. Types, according to method of action

    a. Calcium binders

      (1) Oxalates

        (a) Sodium oxalate

        (b) Heller and Paul's double oxalate (ammonium and potassium)

          1. Dry at not greater than 80°C

          2. 2 mg/ml

      (2) EDTA (Sequestrene): popular

      (3) Citrates: e.g., sodium citrate (coagulation studies)

    b. Anti-thrombins: heparin (natural, for heart therapy)

D. Videotape - The Venipuncture (VT 10, 10:00 min)

  1. Points to be observed (see ANNEX A)

    a. Technique for collection of blood

    b. Preparation of puncture site

    c. Application of tourniquet

    d. Selection of a suitable vein

    e. Disposition of used equipment

  2. Showing of videotape (10:00 min)

E. Questions From Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Principle

2. Techniques

3. Anticoagulants

B. Closing Statement: Knowing the steps for venipuncture will certainly bring the technician nearer to proper execution of the procedure. But part of the success will come from a confident, concerned technician who is meticulous regarding the details of safety. Remember too, as the technician, you must treat the patient as you would want to be treated if you were undergoing a venipuncture.

ANNEX A
INSTRUCTIONAL AIDS

VIDEOTAPE

VT 10, 10:00 minutes - The Venipuncture. The correct technique for the collection of blood from a vein is demonstrated in this videotape. This includes preparation of the puncture site, application of a tourniquet, entering a suitable vein, precautions to avoid a hematoma, transfer of specimens to suitable container, and disposition of used equipment.

NOTE: This videotape is shown in part II.D.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *IM 08-280-007
LABORATORY SCIENCES DIVISION 046
CLINICAL MICROSCOPY BRANCH

Venipuncture

In the 17th century, the artist, Christopher Wren and a chemist, Robert Boyle, performed the first procedure for entry into a living blood vessel. Their procedure utilized a quill attached to a primitive syringe. When Boyle and Wren operated on a dog, the quill was inserted through a lesion made in a blood vessel by a lancet. During this exercise, you will learn all of the precautions and techniques for performing the modern equivalent of this venipuncture, as well as the various anticoagulants used in this and other tests. It is a delicate surgical procedure, requiring the technician's fullest attention and skill.

The most common means of obtaining blood consists of penetration of a blood vessel to obtain a peripheral blood sample. The term "peripheral blood" refers to the particular anatomical area from which the blood is collected: from the external area of the arms or legs, as opposed to the visceral organs, where blood composition differs.

Venipuncutre will be discussed according to two distinct, yet similar techniques: the syringe method, and the Vacutainer method. For the syringe method, a hollow hypodermic needle fits onto a syringe. The needle is usually 19-23 gauge. (Gauge refers to internal diameter; the smaller the number, the greater the diameter). The needle tip is beveled, and the needle must be kept sterile. The usual syringe will contain 10 cc of blood (larger sizes may be used if greater quantities of blood are required) and, like the needle, is disposable. (Formerly, reusable glass syringes and all-metal needles were utilized extensively. They are not so popular now because of the necessity of re-sterilization following use). A tourniquet (often a piece of thin rubber tubing) is used to distend the patient's veins. Seventy percent isopropyl or ethyl alcohol and gauze squares or cotton balls are used to cleanse the site of puncture. The blood is collected in the syringe and subsequently transferred to the proper collection tube, with label. The Vacutainer collection tubes are color-coded for anticoagulant content. The proper tube required for the particular tests to be run on the blood sample must be chosen and labelled prior to puncture. (Discussion of various anticoagulants follows the venipuncture discussion).

The procedure for a syringe venipuncture is not complicated. Yet there are many details to be attended to during the course of those few minutes. First, the technician must cleanse his/her hands thoroughly, and insure that the table area where blood is to be drawn is neat and clean. Assemble the equipment listed above and have it conveniently arranged so that the technician

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*This instructor's manuscript supersedes IM 08-280-007, 014.

can use it without an awkward effort. This is important when the needle is in the patient's arm and the technician must prepare to extract it. A long reach with one arm while attempting to hold the needle stationary can be a traumatic experience for the patient. At the time of equipment assembly, be sure to withdraw the plunger of the syringe to break the seal from manufacture; then re-seat it. (If a re-usable glass syringe is used,be sure the plunger fits the barrel, and that there is no water in either part). Attach the needle without removing the protective cover in such a way that the bevel plane is on the side of the syringe where the graduations show. This allows the technician to later observe the quantity of blood being collected. After removing the cover, the technician must insure also that the needle tip is not burred.

When the equipment and area are properly arranged, the technician assumes a standing position during phlebotomy. Greet the patient cheerfully, yet confidently and professionally to reassure him. Some patients have serious emotional reactions to this procedure, and it is advantageous to minimize the possibility of such a reaction interfering with a successful venipuncture. Have the patient sit in a comfortable chair with one arm resting on the table.

In selecting the site for venipuncture, it is important to note that not every patient has clearly visible veins. Having the patient hang his/her arm down for two or three minutes may help. Even then, some do not have visible vessels. Therefore, this part of the procedure must not be brushed over lightly. Using a quick-release slip-knot, place the tourniquet above the elbow of the patient's most used arm tightly enough to stop venous flow but not arterial flow. The area of choice is the antecubital fossa, i.e. on the inside of the arm, opposite the elbow, where several veins course. The antecubital vein is the most frequent choice, for it is large and associated with very few nerves. Be careful not to stretch the rubber tubing of the tourniquet against the skin, since it tends to pinch. Have the patient make fists, and place one under the elbow of the arm to be punctured for support. Then locate a likely vein for venipuncture. Choose one that is visible, preferably, and of adequate size. Whether the vein is visible or not, always palpate the intended site to insure that you know the exact location, size, depth, and direction of the vein. Use the fingers instead of the thumb to accomplish this, pressing lightly up and down. A vein will spring back, whereas a tendon will remain hard and firm. On a patient with no visible veins, mentally mark the spot where you intend to puncture, using natural skin marks for guides. Be sure you know the size, depth, and direction of the vein to be used. You will not be able to touch the site after it has been cleaned, so it pays to take your time. However, the tourniquet should not be left on the patient's arm longer than two minutes at any one time. Hemoconcentration will occur when the tourniquet is left on for extended periods, which will cause excessive aleration of test results. It should be pointed out that there are other possible sites for venipuncture, such as the back of the wrist, the ankle, and even more unusual sites. These will be used uncommonly, and at the discretion of the attending physician. Small children, burn patients, and the elderly are the most frequent subjects for such treatment.

Now, release the tourniquet. Without contaminating the surface of an alcohol-soaked gauze pad, apply it to the site for venipuncture. Scrub away dirt and oil with either an outward-expanding spiral motion, or parallel linear strokes. In both cases, the gauze should not touch an area more than once. If necessary, cleanse the site again. Allow to air dry, and replace the tourniquet.

You are now ready to make the puncture. Be careful and be aware, for you are dealing with a human body. Grasp the syringe in your most dexterous hand, with the fingertips near the front of the syringe. The most stable position may be with the thumb on top or one side and the fingers on the other. Face the needle bevel up. Have the patient make a fist while you use the thumb of your other hand to anchor the vein. This is very important, for some veins have a decided tendency to slip out of the way when pressure is applied with the needle. Anchor it by placing the thumb on the vein one to two inches below the site of puncture, pulling distally (toward the patient's hand). Without hesitation, make a smooth puncture into the vein above the anchoring thumb in a direction up the arm toward the shoulder (the way the blood flows in the vein). The proper angle to use in making this entry is usually about 30°, depending on the depth of the vein. When the tip of the needle enters the lumen of the vein, there will be a slight "give". At this point, lower the angle and "thread" the needle into the vein for an additional 1/4 to 1/2 inch. This insures a good flow and the needle will not slip out of the vein.

Blood may automatically appear in the hub of the syringe if the patient is a vigorous type and if the puncture is accurate. If it does not, remove the anchoring hand from the patient's arm (NOTE: NOT the hand that made the puncture) and retract the plunger on the syringe, being careful not to withdraw the needle from the vein in the process. One way to prevent this is to lay the back of the hand holding the syringe on the patient's forearm for stability. Aspirate the blood at a moderate rate with the anchoring hand only, again with care not to withdraw the needle. Usually about 7cc will be the amount needed, as this is the amount that will easily fit into an EDTA Vacutainer collection tube. When this amount is collected, release the tourniquet with the anchoring hand (make sure the end doesn't slap the patient in the face). Once again, do not change hands! Also, be careful not to move the needle in the patient's arm. Remember, the tourniquet must never be left on more than two minutes and it must be removed before removing the needle, to prevent unnecessary pain and bleeding.

Under certain circumstances, it may be necessary to draw more blood than the syringe will hold. If Vacutainer systems are not available, and instead of repeating the venipuncture an "exchange syringe" technique is recommended. When an exchange syringe is necessary, a towel should be placed under the patient's arm. A few gauze pads should be placed just in front of the puncture site. The additional syringes are placed next to the patient's arm. The first syringe is filled. Carefully remove the syringe from the needle and hand it to another technician for disposition into the appropriate containers. Carefully place the second syringe on the needle and begin aspirating to fill it. Other syringes, if necessary, are filled in the same manner.

If a second or third syringe is to be used, the needle must lie well within the lumen of the vein. It is expected that a few drops of blood will fall from the hub of the needle during the actual exchanging of the syringes. This is normal and should be explained to the patient prior to performing the procedure. If difficulty is encountered in attempting to remove the syringe from the needle, a second venipuncture is in order.

Lay a piece of clean gauze over the junction of the needle and skin, while quickly pulling the syringe and needle straight back, out of the arm. Apply pressure on the site after withdrawal, and ask the patient to continue the pressure with the arm elevated and extended straight until the bleeding stops.

The blood in the syringe may be transferred to the collection tube in one of two ways. First, remove the stopper from the tube and the needle from the syringe, and slowly expel the blood down the side of the tube. Or, secondly, if a Vacutainer tube is being used for collection, merely push the needle through the rubber stopper and allow the vacuum to withdraw the blood automatically until the tube is full (do not force more than it can take, or cells will be hemolyzed). The first method is the more accurate, for it is gentler to transfer the blood slowly with little pressure, though it is more time-consuming. Immediately re-cap the tube and mix the blood by inversion for about 30 seconds if anticoagulant is being used. Make sure all of the anticoagulant is mixed.

The technician should observe the patient for reaction during this time, as well as during the puncture itself. The most common reaction is fainting (syncope). In this case, the needle must be removed from the arm immediately, the patient should be laid down with feet elevated, and an ammonia ampule crushed under the nostrils of the patient. Loosening tight clothing and providing adequate ventiliation may help. It is also important to remove the patient from the view of other patients at such a time. Another common patient problem is hematoma, or subsurface bleeding due to poor puncture, or insufficient direct pressure following puncture (not to mention excessive probing by the technician). Prevent this by minimizing trauma during the puncture (do not probe excessively), and by removing the needle carefully, applying adequate pressure to the site following puncture. If hematoma appears during the collection, remove the needle immediately since hematomas may be painful.

Convulsions and hysteria may occasionally be encountered during venipuncture. The technician must act quickly and calmly, withdrawing the needle from the arm and restraining the patient from self-injury. Obtain aid immediately.

When the procedure is completed, discard your equipment by bending the needles and breaking the syringe tip (only when using disposable syringes). Discard needles in the special container provided and syringes in wastebaskets.

The Vacutainer method employs the same essential principle of blood collection with a hollow, beveled needle, but replaces the syringe with an evacuated collecting tube. A reusable plastic needle holder guides the vacuum tube onto the rear of the needle. Only 20 gauge needles are used, and must have a double point: one for the puncture at one end, and the other for entry into the collecting tube through the rubber stopper. A tourniquet, gauze, and alcohol must be on hand as in the syringe method.

The Vacutainer method differs from the syringe method in two areas only: assembly of equipment, and aspiration of blood. Before greeting the patient, the double-pointed needle must be screwed firmly onto the Vacutainer holder, with the smaller end inside the holder and the longer end still protected by its plastic guard. Then, fit the proper Vacutainer collection tube (color-coded stoppers indicate the contents) into the holder and onto the needle to the mark molded into the plastic at the front of the holder. Note that there are no calibrations on the holder; the vacuum in the tube controls the amount of blood collected.

Proceed in exactly the same way as with the syringe method to the point of aspiration, cleansing and entering the puncture site carefully. With the Vacutainer method, the anchoring hand at this point should be removed from the patient's arm and used to carefully push the Vacutainer tube fully onto the rear needle in the holder. It is of utmost importance that the needle in the patient' arm not be moved, because the force being applied is in the direction of sensitiv tissues. Allow the blood to fill the tube. Once this is complete, proceed as before in the syringe method. The final exception concerns transfer and mixing of the specimen. Because the blood was collected in the tube in which it will remain, there is no transferral. The mixing may be accomplished by merely extracting the tube from the holder and needle and mixing by repeated inversion for about 30 seconds. Discard the needle; save the holder for reuse, and observe the patient for reaction.

Because of all these details, there are many important sources of error. Technician's difficulties may range from poor selection of the site for puncture to excessive nervousness (due to lack of confidence and experience). Other mistakes to avoid: failure to break the syringe seal and align the bevel with the calibrations in the syringe method; failure to anchor the patient's vein properly; changing hands to aspirate blood; complete transfixion of the vein during entry (be alert for the "give" signal when entering a vein); orientation of the needle bevel in such a way that it is blocked by the wall of the blood vessel; removal of the needle from the vein while retracting the syringe plunger, or pushing the needle into the arm while pushing in the Vacutainer tube; failure to remove the tourniquet before removing the needle from the patient's arm; leaving the tourniquet on longer than two minutes; forcing the blood from a syringe into an evacuated tube; and failure to mix the blood with the anticoagulant immediately after collection if such is required. All of these are well within the control of the technician.

The patient may present difficulties that are not easily overcome even by the experienced technician. The patient, especially if a child, may be unruly and uncooperative, quite willing to fight the technician rather than undergo the procedure. Extra assistance may be required if verbal restraint does not prove satisfactory. Many females and some males have small or obscure veins. Obese people may have deep and unpalpable veins. Some individuals have poorly anchored veins, while the aged may have such scarred (sclerotized) veins that they are difficult to penetrate. Cold temperatures may make veins more difficult to locate, as may circulatory collapse (shock) in emergency patients. All of these are significant problems that may make it quite difficult to draw blood. By following the steps outlined above, and with experience, a technician will eventually be able to perform a successful venipuncture on most anyone. If not, the attending physician should be consulted, as there are other ways of obtaining blood (femoral puncture, jugular puncture, capillary puncture). Venipuncture is often preferable to capillary puncture because of the large amount of blood obtained, allowing numerous tests to be performed in the laboratory away from the patient's bedside.

The blood that has been collected by venipuncture may now be used for hematological testing. Many tests require that the blood be anticoagulated, that is, the blood must not be clotted. This is achieved by using a chemical additive called an anticoagulant. The blood is then referred to as an anticoagulated specimen, containing cells and liquid plasma. (In contrast, the liquid portion of clotted blood is called serum).

Anticoagulants may be subdivided according to the method by which they function. Some are calcium binders. These chemicals, when added to the blood, absorb all of the calcium in the blood. Because calcium is essential for blood clotting, its absence prevents clotting. There are three classes of calcium binders we will discuss briefly: oxalates, citrates, and Sequestrene.

Examples of the oxalate class of anticoagulant are sodium oxalate, and Heller and Paul's double oxalate. The former is used for some coagulation tests and comes in a black-stoppered Vacutainer tube. Heller and Paul's double oxalate is past its prime, but may still be used for certain tests (coagulation work). Heller and Paul's is made of a mixture of ammonium and potassium oxalates, one of which tends to shrink the size and distort the shape of the red blood cells, while the other has the opposite effect. The two together cancel each others' effects, maintining the red blood cells' normal size and

and shape. When it is used, it must be used in a ratio of 2mg to 1 ml of blood. To dry the powder form, it must be heated at not greater than 80° C, for the oxalate will oxidize to carbonate, which is of no use. Heller and Paul's double oxalate causes disintegration of the white blood cells within a few hours. For this reason, this anticoagulant is not the reagent of choice for white blood cell counts or differentials, and in fact is seldom used today.

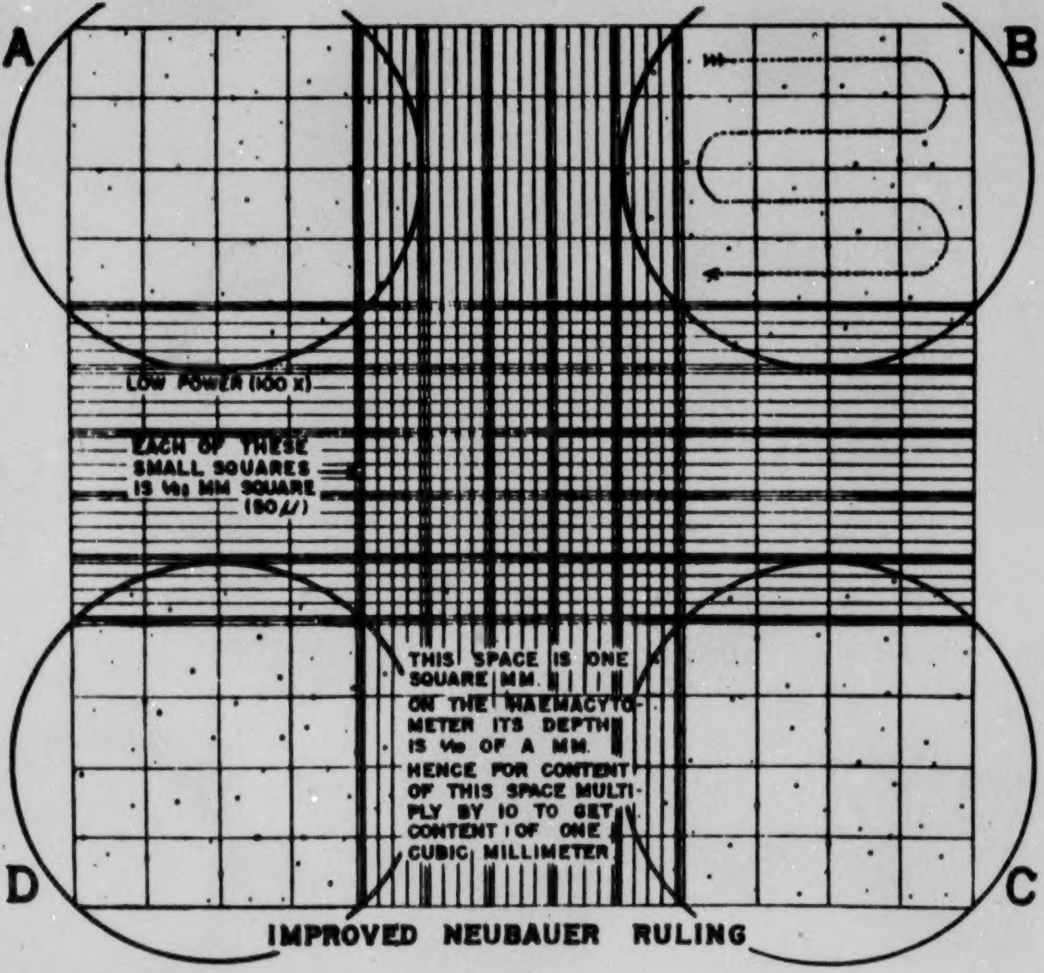
A second class of calcium binders is the citrates. Sodium citrate is a common example, used often in coagulation studies. Another citrate is used extensively in blood banking because of its low toxicity to the human system. Citrates come in yellow-topped Vacutainer tubes. A third class of calcium binder is Sequestrene, or EDTA (a salt of ethylene diamine tetracetic acid). This is currently the most popular anticoagulant for general use. The white blood cells are preserved adequately for differential counts for up to 12 hours and for cell counts even longer. However, it is not applicable to some laboratory tests, especially certain coagulation studies and some chemistry procedures. EDTA should be used in a ratio of 1-2 mg/ml of blood, and is found pre-measured in purple top Vacutainer tubes as a white powder.

Blood may be anticoagulated by means other than binding of calcium. The only type of anticoagulant we will discuss here is the anti-thromboplastin and anti-thrombin called heparin. This is a natural compound found in the granules of basophils and in tissue cells called mast cells. Because of its low toxicity, it is commonly used as a therapeutic measure for patients with coronary conditions due to thrombosis (spontaneous clot formation). However, it mixes poorly with the blood in a collecting tube, and leaves a blue background when the blood is Wright's stained and smeared. Therefore, its use is limited in the laboratory. It is placed into capillary tubes that are used for hematocrit determinations and blood collection for smear-making.

Knowing the steps for venipuncture will certainly bring the technician nearer to proper execution of the procedure. But part of the success will come from a confident, concerned technician, who is meticulous regarding the details of safety. Remember too, as the technician you must treat the patient as you would want to be treated if you were undergoing a venipuncture.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-007-1
LABORATORY SCIENCES DIVISION 014
CLINICAL MICROSCOPY BRANCH

IMPROVED NEUBAUER RULING


A
B
LOW POWER (100 X)
EACH OF THESE SMALL SQUARES IS 1/20 MM SQUARE (50 μ)
THIS SPACE IS ONE SQUARE MM. ON THE HAEMACYTOMETER ITS DEPTH IS 1/10 OF A MM. HENCE FOR CONTENT OF THIS SPACE MULTIPLY BY 10 TO GET CONTENT OF ONE CUBIC MILLIMETER
D
C
IMPROVED NEUBAUER RULING

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*This mimeo supersedes M 08-280-007-1, 041.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
CLINICAL MICROSCOPY BRANCH

*M 08-280-007-3
054

Homework on Blood Dilutions

A. Blood dilutions for White Blood Cell counts:

	BLOOD DRAWN TO THE:	DILUTION FACTOR:	CELLS COUNTED ON HEMACYTOMETER:	RESULTING TOTAL CELL COUNT:
1.	0.7 mark	1:_____	51, 49, 47 & 53	___________WBC/cmm
2.	1.0 mark	1:_____	58, 41, 55 & 46	___________WBC/cmm
3.	0.4 mark	1:_____	48, 51, 54 & 47	___________WBC/cmm
4.	0.1 mark	1:_____	45, 51, 47 & 57	___________WBC/cmm
5.	0.5 mark	1:_____	54,53 , 48 & 45	___________WBC/cmm

NOTE: Round all total WBC counts to the nearest 50 cells

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*This mimeo supersedes M 08-280-007-3, 012

B. Blood dilutions for Red Blood Cell counts:

	BLOOD DRAWN TO THE:	DILUTION FACTOR:	CELLS COUNTED ON HEMACYTOMETER:	RESULTING TOTAL CELL COUNT:
1.	0.5 mark	1:_____	101, 110, 98, 100 and 91	__________RBC/cmm
2.	1.0 mark	1:_____	97, 102, 98, 109 and 94	__________RBC/cmm
3.	0.1 mark	1:_____	105, 98, 100, 102 and 95	__________RBC/cmm
4.	0.3 mark	1:_____	100, 101, 97, 101 and 101	__________RBC/cmm
5.	0.8 mark	1:_____	98, 99, 100, 101 and 102	__________RBC/cmm

NOTE: Round all total RBC counts to the nearest 10,000 cells

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SFC Wilburn Tanne
*LP 08-280-008
048

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
CLINICAL MICROSCOPY BRANCH

Blood Dilutions and Cell Enumerations(2 hours)

COURSE PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic)

PLACE: Laboratory

REFERENCES:

TM 8-227-4

STUDY ASSIGNMENT: Study TM 8-227-4, pp 5-1 to 5-8 after class.

STUDENT UNIFORM AND EQUIPMENT: Not Applicable

TOOLS, EQUIPMENT, AND MATERIALS: M 08-280-007-1 issued during lecture; M 08-280-008-1 issued during lecture; M 08-280-007-3 issued after class.

PERSONNEL: One primary instructor (MOS 92B3H or 92B4H).

INSTRUCTIONAL AIDS: Chalk, chalkboard, eraser, overhead projector and transparencies.

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

FIRST HOUR

I. INTRODUCTION (10 min)

A. Opening Statement: The original discovery of cellular elements in the blood came at a time when little was known of disease processes and laboratory studies. At present, however, research in numerous fields has uncovered association between disease and alterations in blood composition. Some of the significant changes in composition are related to variations in the number of cells found in the blood. During this lecture, you will learn how to perform both white and red blood cell counts. While these counts are usually not diagnostic in themselves, they may be valuable indicators of disease to the physician. However, these counts are intricate and replete with sources of error. Therefore, you must be aware of these pitfalls, and diligent in your procedural techniques to fulfill your future role as a technician.

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*This lesson plan supersedes LP 08-280-007, 075

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LP 08-280-008
048

B. Objectives

1. List the proper steps for performing both red blood and white blood cell dilutions and enumerations.

2. List the units of report, and normal values for both the red blood and white blood cell enumerations.

3. Given the necessary data, calculate the red blood and white blood cell count to the nearest whole number to include the correct unit of report.

C. Class Procedure and Lesson Tie-in

1. 35 minute slides demonstrating these procedures to follow.

2. Blood dilutions and cell enumeration practice to follow.

3. Blood cell enumeration to be used as a regular part of the CBC exercises performed as a crucial part of hematology instruction.

II. EXPLANATION (65 min)

A. Blood Dilutions

1. RBC

a. Principle

b. Equipment

(1) RBC diluting pipet
(2) Aspirator
(3) Gauze or tissue

c. Reagents

(1) Functions

(a) Dilution
(b) Preservation of RBC

(2) Types

(a) Hayem's solution
(b) Normal (0.85%) physiological saline

d. Procedure

(1) Prepare specimen and equipment
(2) Aspirate blood (normally to 0.5 mark) and wipe pipet
(3) Aspirate diluent (normally to 101 mark) and swivel
(4) Remove aspirator tubing and mix
(5) Calculate dilution factor

(a) Formula: $\frac{\text{Total volume of bulb}}{\text{Volume of blood used}}$ = dilution factor

(b) Round to nearest 0.1
(c) Standard RBC dilution factor = 200

e. Sources of error

(1) Dirty or wet pipets
(2) Diluent containing foreign matter
(3) Poor loading and wiping of pipet
(4) Bubble in pipet
(5) Clot in pipet

QUESTION: Will a clot in an RBC pipet cause a raised or a lowered count?

ANSWER: Lowered, due to red cells being tied up in the clot.

2. WBC

a. Principle

b. Equipment

(1) WBC diluting pipet

NOTE: Draw diagrams on board, explaining the pipet.

(2) Aspirator
(3) Gauze or tissue

c. Reagents

(1) Functions

(a) Dilution
(b) Lysis of RBC
(c) Preservation of WBC

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(2) Types

(a) HCl, 0.1N
(b) Acetic acid, 2%

d. Procedure

(1) Prepare specimen and equipment
(2) Aspirate blood (normally to 0.5 mark) and wipe

QUESTION: What cases would call for other than normal dilutions?

ANSWER: Unusually high or low numbers of cells.

(3) Aspirate diluent to 11 mark and swivel
(4) Remove aspirator and mix
(5) Calculate dilution factor

$$\text{Formula: } \frac{\text{Total volume of bulb}}{\text{Volume of blood used}} = \text{dilution factor}$$

QUESTION: What is the standard dilution factor?

ANSWER: Standard WBC dilution factor: 10/0.5 = 20.

e. Sources of error

(1) Dirty or wet pipet
(2) Particulate matter in diluent
(3) Poor loading and wiping of pipet
(4) Bubble in pipet
(5) Clot in pipet

SECOND HOUR

B. Cell Enumeration

1. RBC

a. Principle: Counting and calculation of RBC/mm^3 blood

b. Equipment

(1) Hemacytometer and coverslip
(2) Handcounter
(3) Microscope

c. Procedure

(1) Prepare equipment

(2) Mix cells in RBC pipet
(3) Discard 4 drops, wiping tip dry with finger
(4) Load hemacytometer smoothly(entire raised area covered, no run-over into moat)
(5) Let settle 3 minutes
(6) Count RBC's (translucent disks)

(a) Under high dry power
(b) Four corners and center of center mm^2
(c) Rule of cells touching sides

(7) Calculate

(a) Factors

1. Dilution factor (normally 200)
2. Area factor (normally 5)
3. Depth factor (normally 10)

(b) Formula:

$$RBC/mm^3 = \text{No. of cells counted} \times \text{depth factor} \times \text{area factor} \times \text{dilution factor}$$

(c) RBC standard K factor = 10,000

QUESTION: What is the value reported to the physician for a red count of 438 performed with standard dilution?

ANSWER: 4,380,000 RBC/mm^3.

(d) Sources of error

(1) Dirty or scratched hemacytometer
(2) Improper loading of hemacytometer
(3) Counting artefacts

(e) Normal values:

Adults

(1) Females: 3.6 to 5.0 million RBC/mm^3
(2) Males: 4.2 to 5.4 million RBC/mm^3

2. WBC

a. Principle: counting and calculation of WBC/mm^3 blood

b. Equipment

(1) Hemacytometer and coverslip

(2) Handcounter
(3) Microscope

c. Procedure

(1) Prepare equipment
(2) Mix cells in WBC pipet (3-5 min by hand)
(3) Discard 3 drops
(4) Load hemacytometer smoothly (entire raised area covered, no run-over into moat)
(5) Let cells settle 3 minutes
(6) Count WBC's

(a) Under low power
(b) Four corner mm^2
(c) Maximum difference between corners: 10 cells
(d) Observe rule of cells touching sides

(7) Calculate

(a) Factors

1. Dilution factor (normally 20)
2. Area factor (normally 1/4)
3. Depth factor (normally 10)

(b) Formula:

$$WBC/mm^3 = \text{No. of cells counted} \times \text{Depth factor} \times \text{Area factor} \times \text{Dilution factor}$$

(c) WBC standard K factor = 50

QUESTION: What is the value reported to the physician for a white count of 118 performed with standard dilution?

ANSWER: 5,900 WBC/mm^3

QUESTION: What is the value reported to a physician for a white count of 264 counted in $4mm^2$ with blood drawn to the 0.7 mark of the WBC pipet?

ANSWER: 9,450 WBC/mm^3, rounding dilution factor to nearest 0.1

(d) Sources of error

(1) Improper shaking of pipet
(2) Failure to wipe drop from pipet tip
(3) Dirty or scratched hemacytometer
(4) Improper loading of hemacytometer
(5) Counting artefacts

e. Normal values:

Adults: 4,500 to 11,500 WBC/mm^3

C. Automatic Cell Counters

NOTE: Inform students of their study assignment in TM 8-227-4, pp 5-1 to 5-8.

D. Questions from Students

III. SUMMARY (5 min)

A. Review of main points

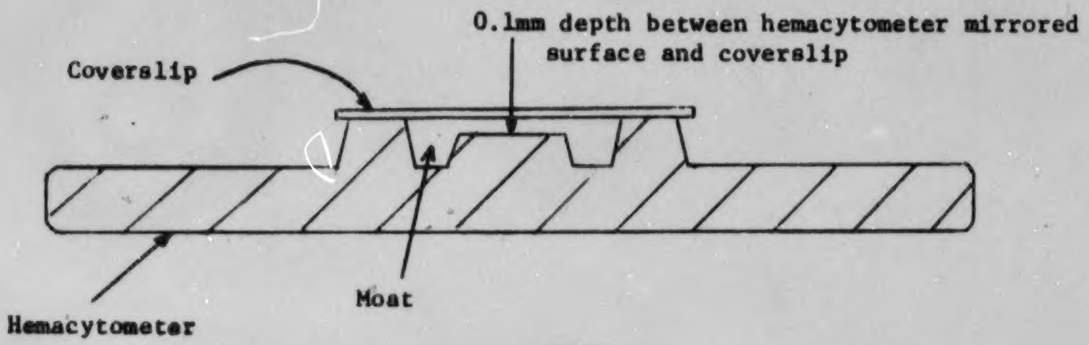
1. Blood dilution

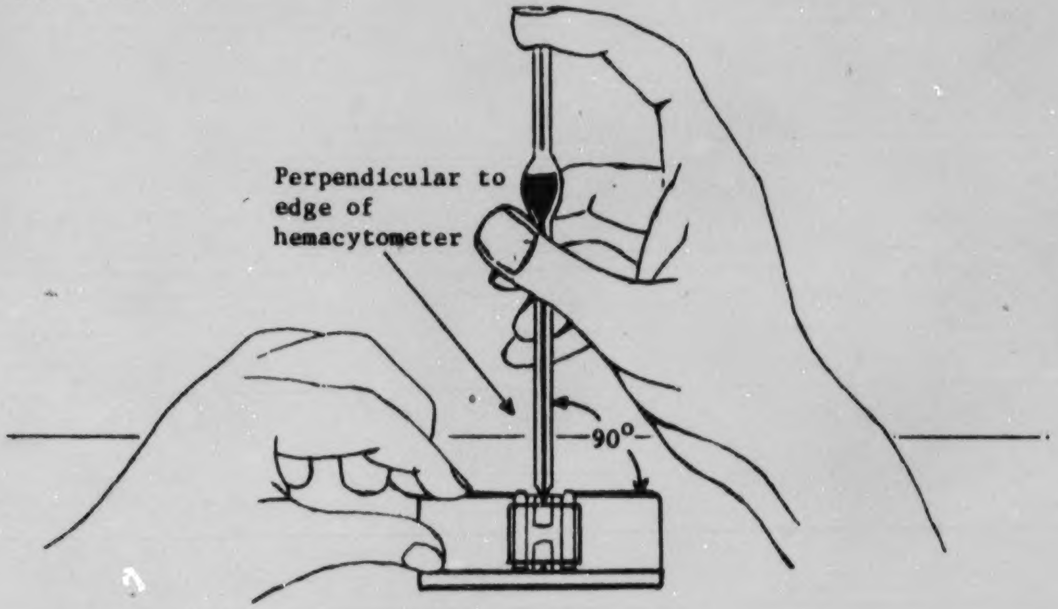
2. Cell enumeration

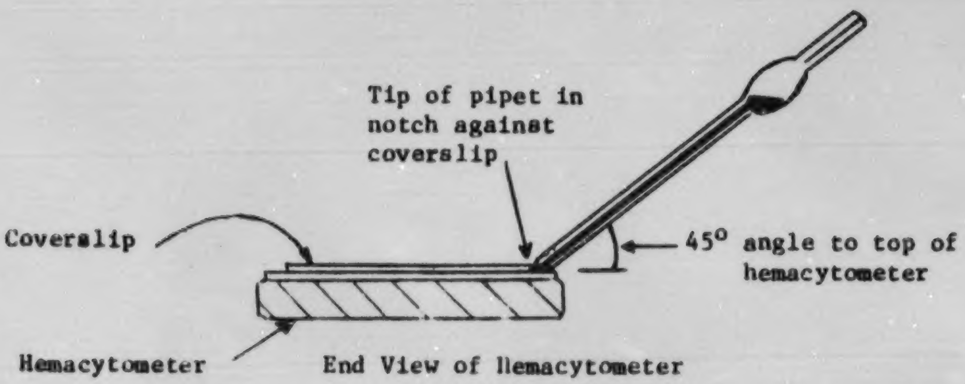
B. Closing Statement: In practicing these procedures of dilution and enumeration, the technician will struggle at times to remember the proper methods and to train himself to carry out these methods. It will require considerable attention to detail and practice, especially with the white blood cell count, which is routinely performed. Achievement of mastery will be worth the effort expended.

Method for Charging Hemacytometer Counting Chamber

Side View of Hemacytometer


0.1mm depth between hemacytometer mirrored surface and coverslip
Coverslip
Moat
Hemacytometer


Perpendicular to edge of hemacytometer
90°


Tip of pipet in notch against coverslip
Coverslip
45° angle to top of hemacytometer
Hemacytometer

End View of Hemacytometer

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY ***LP 08-280-011**
LABORATORY SCIENCES DIVISION 105
CLINICAL MICROSCOPY BRANCH

Capillary Puncture and Hemoglobin Determination (3 hours)

COURSES PRESENTED TO: 311-92B20, Medical Laboratory Procedures (Basic)

PLACE: Laboratory

REFERENCES:

TM 8-227-4
Davidsohn, I. and Henry, J. B. Todd-Sanford Clinical Diagnosis by Laboratory Methods. 14th ed. Philadelphia: W. B. Saunders, 1969.
Miale, John B. Laboratory Medicine Hematology. 3rd ed. St. Louis: C. V. Mosby, 1967.
Technical Methods and Procedures of the American Association of Blood Banks. 5th ed. Chicago: American Association of Blood Banks, 1970.
White, Abraham; Handler, Phillip; and Smith, Emil L. Principles of Biochemistry. 4th ed. New York: McGraw-Hill, 1968.
Wintrobe, Maxwell M. Clinical Hematology. 6th ed. Philadelphia: Lea and Febiger, 1967.

STUDY ASSIGNMENT: Study TM 8-227-4, pp. 3-5 to 3-8 and 5-18 to 5-20 after class

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: None

PERSONNEL: One primary instructor (MOS 92B3H or 92B4H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, eraser, TV receivers, and Videotapes #11 & 287. Lapel microphones, etc.

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

FIRST HOUR

I. INTRODUCTION (7 min)

A. Opening Statement: At certain times, because of age or condition of the patient, a venipuncture is not practical. When only small volumes of blood are required, the specimen will be procured by

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*This lesson plan supersedes LP 08-280-011, 084

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means of a capillary puncture. This is a more controlled procedure than pricking oneself with a pin, yet it is almost as simple. Among other tests, the hemoglobin determination requires such a small amount of blood that the specimen may be easily and quickly obtained from the capillary bed. This is fortunate, since the hemoglobin determination is one of the most commonly requested tests in the clinical laboratory.

B. Objectives

1. List and explain the proper steps in performing a capillary puncture.

2. Name five forms and three genetic types of hemoglobin.

3. Classify the four hemoglobin determinations discussed during this lecture as gravimetric or colorimetric, and direct or indirect, and as visual or photometric methods.

4. List and explain the proper steps in performing a hemoglobin determination by the cyanmethemoglobin technique.

5. State the normal values of hemoglobin concentration for adults.

C. Class Procedure and Lesson Tie-In

1. Capillary puncture practical exercise follows the lecture and is a regular CBC technique exercise.

2. Hemoglobin determination by the cyanmethemoglobin technique is included as part of the practical exercise.

3. Videotapes demonstrating capillary puncture and hemoglobin determination to be included in this lecture.

4. Graded exercises for capillary puncture technique and cyanmethemoglobin determination at the close of hematology.

II. EXPLANATION (110 min)

A. Capillary puncture

1. Principle

a. Advantages

(1) Less patient apprehension

(2) Simple and quick technique

- b. Disadvantages
  - (1) CBC only
  - (2) Tissue fluids
- 2. Equipment
  - a. Alcohol (70 percent isopropyl or ethyl)
  - b. Gauze
  - c. Lancet (Hemolet): 4mm length

NOTE: Draw picture of lancet on chalkboard.

- 3. Procedure
  - a. Choose site for puncture
    - (1) Lateral aspect of middle two fingertips (site of choice)

NOTE: Draw picture of finger showing proper area for puncture.

QUESTION: Why is the lateral aspect of the finger usually chosen for puncture?

ANSWER:

- (a) Callous, fat, located on ball of finger
- (b) Less pain for patient

    - (2) Other areas
      - (a) Ear lobe (painless, but cold)
      - (b) Heel (newborns)
      - (c) Great toe (infants)
  - b. Prepare site for puncture
    - (1) Warm site
    - (2) Cleanse vigorously (air dry)
    - (3) Engorge fingertip with blood; hold firmly

c. Puncture with lancet

(1) Perpendicular to fingerprint

(2) Quick, firm stab

(3) Three to four millimeters deep

QUESTION: Is it more painful to stab three or four times improperly than once properly?

ANSWER: Yes, it is more painful.

d. Wipe away first drop (tissue fluids)

4. Sources of error

a. Failure to puncture lateral aspect of tip

b. Failure to puncture perpendicular to print

c. Failure to puncture three to four millimeters deep

B. Hemoglobin determinations

QUESTION: What gives blood its red color?

ANSWER: Hemoglobin, the primary component of red blood cells.

1. Hemoglobin chemistry

a. Primary functions

(1) Transports oxygen (O_2) from lungs to tissues

(2) Aids transport of carbon dioxide (CO_2) from tissues to lungs

(3) Aids in plasma buffering

b. Chemical nature

NOTE: Draw stylized picture of hemoglobin molecule (Annex A) on chalkboard.

(1) Protein portion (globin): 2 alpha, 2 beta chains

(2) Iron-containing portion (heme): 4 heme groups

  (a) One ferrous ion (Fe^{+2}) per heme

  (b) Imparts red color

c. Forms of hemoglobin

  (1) Oxyhemoglobin (O_2 + hgb)

  (2) Reduced hemoglobin (From CO_2 + hgb)

  (3) Carboxyhemoglobin (CO + hgb)

  (4) Sulfhemoglobin (Irreversible)

  (5) Methemoglobin (Fe^{+3})

d. Genetic types of hemoglobin

  (1) Hemoglobin A (adult)

  (2) Hemoglobin F (fetal)

  (3) Hemoglobin S (Sickle cell)

  (4) Others (hemoglobinopathies)

2. Techniques for hemoglobin determinations

  a. Gravimetric method (specific gravity, copper sulfate)

    (1) Principle

    (2) Use: screening blood donors

  b. Colorimetric methods

    (1) Principle

    (2) Visual methods

NOTE: The two well-known types of visual methods for colorimetric hemoglobin determination (the A.O. Spencer, a direct method using saponin to lyse the red blood cells, and the Salhi-Hellige, an indirect method using hydrochloric acid to form acid hematin) are discussed in the videotape which accompanies this lecture (#287).

(3) Photometric methods

(a) Principle

(b) Direct methods (example: oxyhemoglobin; in disuse)

(c) Indirect methods: cyanmethemoglobin technique (Drabkin's)

1 Principle

a Stable cyanide derivative

b Spectrophotometer and Beer's Law

c Popular, standardized technique

d Measures all but sulfhemoglobin

SECOND HOUR

2 Equipment

a Aspirator

b Sahli pipet

(1) No mixing bulb

(2) Contains 20 cmm = 20 mm^3 = 20 lambda = 0.02 ml

c Test tubes, 13 x 100 mm

d Cuvets, 19 x 150 mm

e Spectrophotometer

3 Reagent: Drabkin's solution (contains cyanide)

4 Procedure

a Mix blood specimen

b Pipet 20 cmm blood (wipe clean)

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c Deliver into 5 ml Drabkin's solution

d Read in spectrophotometer

- (1) Set wavelength to 540 nanometers (green light)
- (2) Zero
- (3) Blank with Drabkin's (wipe cuvet)
- (4) Read percent transmittance of sample (wipe cuvet)
- (5) Report in gm Hgb/dl blood, using conversion chart

5 Sources of error

- a Failure to measure blood accurately
- b Failure to wipe pipet carefully
- c Improper use of spectrophotometer

6 Normal values

- a Males: 14-17 gm Hgb/dl blood
- b Females: 12-16 gm Hgb/dl blood

7 Abnormal values

- a Anemia (lowered)
- b Polycythemia (elevated)

C. Videotapes

1. Introduction

NOTE: See Annexes B and C for summary of tapes' contents.

2. Videotape #11: Capillary Puncture (running time: 08:30 min.)

3. Videotape #287: Hemoglobin Determinations (running time: 19:12 min.)

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4. Summary

D. Questions from Students

III. SUMMARY (3 min)

A. Review of Main Points

1. Capillary puncture

2. Hemoglobin determinations

3. Videotapes

B. Closing statement: Without hemoglobin and its strong affinity for oxygen, the human body would require at least 100 times the amount of blood available to maintain itself. In addition to this vital function, molecules of hemoglobin, present in the millions in each red blood cell, are important in acid-base balance and carbon dioxide removal. Therefore, it is obvious why a hemoglobin determination, performed on blood obtained either by a simple capillary puncture or by a venipuncture, is of prime importance to a physician diagnosing anemias and other illnesses. The laboratory technician must perform with skill and accuracy, for his results may profoundly affect the welfare of the patient.

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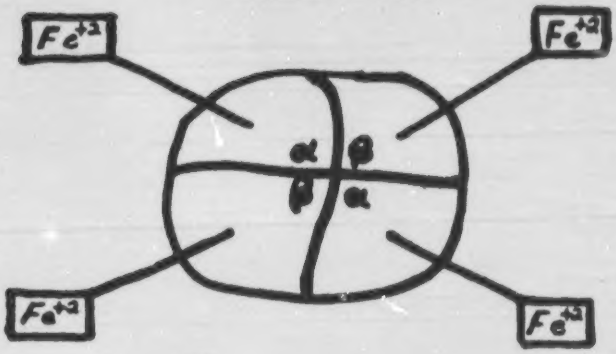
LP 08-280-011
105

ANNEX A

INSTRUCTIONAL AIDS

Chalk Drawing


Fe^{+2}
Fe^{+2}
α
β
β
α
Fe^{+2}
Fe^{+2}

NOTE: Draw on chalkboard at beginning of discussion of II.B.1.b.

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ANNEX B

INSTRUCTIONAL AIDS

Videotape #11 "Capillary Puncture" Running time: 08:30 min.

Collection of blood from the lateral tip of the finger by an approved capillary puncture technique is demonstrated in this videotape. This includes itemizing equipment required, preparation of the site, performing the capillary puncture, collection of representative specimens, and disposition of used equipment.

NOTE: This videotape is to be shown during the second hour, under Part II.C.

ANNEX C

INSTRUCTIONAL AIDS

Videotape #287 "Hemoglobin Determinations" Running time: 19:12 min.

A demonstration of the three commonly used clinical laboratory methods for determination of hemoglobin are given: (1) The Salhi-Hellige, indirect visual method; (2) The cyanmethmoglobin, indirect spectrophotometric method; (3) The A.O. Spencer Hemoglobin Meter, direct visual method.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *IM 08-280-011
LABORATORY SCIENCES DIVISION 105
CLINICAL MICROSCOPY BRANCH

At certain times, because of age or condition of the patient, a venipuncture is not practical. When only small volumes of blood are required, the specimen will be procured by means of a capillary puncture. This is a more controlled procedure than pricking oneself with a pin, yet it is almost as simple. Among other tests, the hemoglobin determination requires such a small amount of blood that the specimen may be easily and quickly obtained from the capillary bed. This is fortunate, since the hemoglobin determination is one of the most commonly requested tests in the clinical laboratory.

At times, for one reason or another, the capillary puncture must be used to obtain the necessary blood specimen. However, one should not feel that this procedure is used only when blood can be obtained in no other way. There are definite advantages to the use of the capillary puncture as a matter of routine. It is simple to perform and can be done by relatively untrained personnel; it is quite rapid, requiring little of the patient's or technician's time; and (probably most important) there is usually less patient apprehension than with a venipuncture, although the capillary puncture is just as painful. In addition, because no anticoagulant is used, red blood cell morphology on thin blood smears made from this blood is usually of good quality.

There are some disadvantages to its use. Generally speaking, the amount of blood that can be easily obtained is sufficient only for a routine "complete blood count." If other tests or determinations are requested by the medical officer, a venipuncture is indicated. In addition, by the time specimen analysis has been initiated, usually the patient has left the laboratory. If for some reason the specimen is unsatisfactory--a pipet improperly filled or a smear badly stained--the ambulatory patient must return or the technician must revisit the in-patient. In either case, the capillary puncture must be repeated and considerable technician as well as patient time is lost.

The equipment necessary for the proper performance of the capillary puncture is simple. A sterile lancet or Hemolet with a tip 4mm in length is the puncturing instrument. A needle or blade pushed through a cork must not be used because of the possibility of contamination. A scalpel blade may be used if a lancet is not available provided the technician is always mindful of possible injury to the patient. In addition to the lancet, 70 percent isopropyl or ethyl alcohol and 2" x 2" gauze squares or cotton are needed.

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*This instructor's manuscript supersedes IM 08-280-011, The technique is equally simple. Initially, the site must be selected. Why is the lateral aspect of the tip of the finger usually chosen for puncture? There are several reasons for this choice. The skin over the ball of the finger is usually thicker and more calloused than over the lateral aspect making it more difficult to penetrate the capillary bed to a depth sufficient to obtain a satisfactory flow of blood. Also, the lateral aspect is just as accessible as the ball of the finger; it is not likely to be used as much by the patient so that puncture at this site results in less residual patient discomfort during the healing period. Refrain from puncturing edematous or cyanotic areas.

The primary disadvantage of the lateral site lies in one of the prevailing conditions which often cause the capillary puncture to be chosen over the venipuncture as the method to be used in obtaining blood specimens: the size of the patient. The fingers of infants and small children are usually too small to be held properly.

The lobe of the ear is another site sometimes used for capillary puncture. The ear lobe has the advantage of being poorly supplied with sensory nerve endings; a puncture in this area being almost painless. In addition, this area is usually accessible on any patient, since the ear is seldom covered with dressings (as the hands occasionally may be). However, as with other sites, the ear lobe must be warm before the specimen is obtained. Blood from the ear lobe often contains more lymphocytes than blood from the finger.

Small children and babies do not, as a rule, have sufficient tissue on their finger pads to make the finger a good site for puncture. With these patients, the great toe or the heel must be used. The heel in particular is almost always large enough to furnish several individual sites when repeated punctures must be made (as, for example, when microbilirubins are to be done at timed intervals on an erythroblastotic infant).

Having selected the puncture site, the technician must prepare the area. This is done by cleansing the skin over the chosen site with a 2" x 2" gauze pad or cotton ball wet with 70 percent alcohol. Two goals are accomplished by this step: (1) dirt, fatty debris, and viable bacteria are removed; and (2) the circulation through the capillaries is increased. After cleaning the site, and before performing the actual puncture, sufficient time must be allowed for the skin to dry and the circulation to equalize. Care must be taken not to touch the cleaned area before the puncture.

The cover on the lancet point is removed, and the lancet held between the thumb and the first two fingers. The patient's finger, ear lobe, or foot (in the case of a baby) is held firmly by the other hand with pressure applied to hold the skin taut over the chosen site and engorge the site with blood. The puncture itself is made by a quick, firm stab with the point of the lancet, causing an incision through the skin and into the underlying tissue to a minimum depth of three to four millimeters. This incision

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should be at right angles to the fingerprint whorls to insure that the blood will form a drop and not run down the fingerprint grooves. The motion used to make this incision can be a short, straight thrust, or a rolling motion with the last two fingers of the lancet hand braced against the patient's hand. Experience in performing the puncture will dictate which of these motions is best for you as a technician. After withdrawing the lancet, a drop of blood appears over the incision; this drop will contain some tissue fluid and should be wiped off with the gauze pad. The second and succeeding drops are used to fill the pipets and tubes and to make the thin blood film. Be sure to wipe the finger clean between these procedures to insure that no dried or clotted blood is used. Performed correctly, the incision will be deep enough that blood will flow freely and a sufficient amount can easily be obtained for all the requested tests and determinations. Gentle pressure on the finger, massaging toward the fingertip, but only up to the last knuckle, is permissible to increase the flow. Care must be exercised to squeeze the finger since excessive pressure results in dilution of the blood specimen with fluid from the tissues, leading to inaccuracy in the final report. After the specimen has been taken, a gauze pad (preferably sterile) is placed over the puncture site and pressure applied until the bleeding has ceased.

Failure to puncture perpendicular to the fingerprint lines, failure to select the proper site of the proper finger for puncture, and failure to penetrate the minimum three to four millimeters can completely negate the value of sticking the patient with a lancet. These sources of error can readily be avoided with practice. The patient's comfort and well-being certainly deserve your maximum effort.

What constituent gives blood its red color? The major component of the red cell, hemoglobin, is responsible, but the red color is incidental, as far as we know. Nature could conceivably have made hemoglobin green, as she did with a very similar substance, the chlorophyll of plants. Simply stated, hemoglobin transports oxygen from the lungs to other tissues of the body and, along with the plasma, transports carbon dioxide from the tissues to the lungs. This gaseous exchange is a continuous process, and its interruption for any extended length of time may result in death. Even though death may not follow, interruption of the oxygen-carbon dioxide cycle in man for more than four minutes can often result in irreversible brain damage. Hemoglobin also serves the very important (though less widely known) function of buffering the blood against significant pH changes that could occur as the result of carbon dioxide release from the tissues into the blood. Carbon dioxide combines with water in the erythrocytes to form carbonic acid, which could lower the pH of the blood and tissues by dissociating into hydrogen ions and bicarbonate ions. However, hemoglobin "absorbs" these hydrogen ions and allows the non-acidic bicarbonate ion to diffuse out of the red blood cells into the plasma (balanced by a shift of chloride ions into the cells). The bicarbonate, along with a small amount of carbonic acid, acts as a buffering system in the plasma itself.

Because of its obvious importance to the physical well-being of the patient, hemoglobin concentration is of great interest to the medical officer. For this reason, the hemoglobin concentration is one of the most commonly requested determinations received in the clinical laboratory.

Since the results you report must be accurate and in order to better understand the principles of the procedures used to determine its concentration, the technician must have some idea of the nature of hemoglobin. The hemoglobin molecule is a conjugated protein with a molecular weight of approximately 65,000. It is composed of one molecule of globin (the basic protein portion) and four molecules of heme, each of which contains one atom of iron in the ferrous or reduced form. It is this ferroheme which serves as the active portion of the entire molecule, combining with oxygen or carbon dioxide in a reversible manner without changing ionic states. Heme comprises about four percent of the total weight of the hemoglobin molecule. It is a metal complex consisting of an iron atom in the center of a porphyrin ring. The basic protein, globin, is composed of two pairs of polypeptide chains which have been designated as alpha and beta in the normal individual. These pairs are held together by noncovalent forces to form a three-dimensional globe-shaped protein. Each chain is made up of about 150 amino acids. One heme covalently bonds to each chain (at a histidine residue). The heme resides in a hydrophobic, low-dielectric pocket of the chain through which oxygen may easily penetrate.

There are many different types of hemoglobin, depending on the kinds of protein chains in the globin portion. The most commonly encountered types of hemoglobin are A, F, and S, which are genetically determined. Hemoglobin A, or adult hemoglobin, consists of two alpha and two beta chains in the globin portion. It makes up 98 to 100 percent of the hemoglobin in normal adults. Hemoglobin F, or fetal hemoglobin, comprises from 50 to 90 percent of the normal newborn hemoglobin, an amount which tapers off to adult levels of zero to one percent within a few years. This hemoglobin is capable of carrying oxygen and carbon dioxide as Hemoglobin A. Hemoglobin F contains two alpha and two gamma chains in its globin portion. Hemoglobin S is considered an abnormal hemoglobin, not because of an inability to carry oxygen, but because of its effect on the physical shape of the red blood cell under certain conditions. It is able to carry oxygen and carbon dioxide, but in effecting alteration of erythrocyte shape, it can inhibit or prevent circulation. This hemoglobin S is also called sickle cell hemoglobin, which will be discussed later in the course. Briefly, the S globin is only slightly different from the globin of hemoglobin A (a valine amino acid is substituted for a glutamic acid residue at the sixth position of each beta chain). Nevertheless, the implications of such a change in chemical composition are profound. The cause of this change has been traced to one specific gene in human chromosomes; in this discovery, the frontier of investigation of molecular diseases has been advanced to its very source.

Hemoglobin is normally found in two forms in the circulatory system of the body, depending upon its location in the gaseous exchange cycle. Hemoglobin is transformed into oxyhemoglobin in the lungs where the oxygen tension is high. Four molecules of oxygen bind loosely to the heme portion, changing the shape of the hemoglobin molecule with each oxygen added. This complex is readily dissociable and releases the oxygen when it reaches the body tissues where the oxygen tension and pH are low. (This phenomenon has been called the "Bohr effect," which states that hemoglobin has less affinity for oxygen in low pH environment.) About 95 percent of the hemoglobin reaching the lungs is converted to oxyhemoglobin. After the oxyhemoglobin has released its oxygen in the tissues, the hemoglobin is free to carry carbon dioxide to the lungs. However, only about twenty percent of the carbon dioxide is carried in this form (sometimes called carbamino hemoglobin, so called because the CO_2 is bonded to an amino group of the globin portion of hemoglobin and not to the heme portion). Most of the remaining carbon dioxide to be transported enters the red blood cell, forms carbonic acid which dissociates into hydrogen ions and bicarbonate ions, and returns to the plasma in the form of bicarbonate. When the bicarbonate reaches the lungs, this process is reversed and carbon dioxide is exhaled. The hemoglobin in this process was reduced by the hydrogen ions (at the histidine residues) and is thus spoken of as "reduced" hemoglobin. In addition, a small fraction of the total carbon dioxide load is carried in the blood plasma as carbonic acid, but the majority is carried as bicarbonate. It should be noted that in both oxyhemoglobin and reduced hemoglobin, heme remains in the ferrous state (Fe^{+2}).

These two physiologic forms, oxyhemoglobin and reduced hemoglobin, are readily converted into a series of compounds by the action of acids, alkalies, oxidizing and reducing substances, and other agents. For example, hemoglobin combines with carbon monoxide in the same proportion as with oxygen to form carboxyhemoglobin or HbCO. However, hemoglobin's affinity for carbon monoxide is 210 times greater than for oxygen. As a result, carbon monoxide, even when present in the air in very low concentrations, is bound by the hemoglobin to the exclusion of oxygen. The carboxyhemoglobin cannot bind oxygen, so no oxygen is carried to the tissues. Tissue anoxia is further compounded by the fact that mixtures of carboxyhemoglobin and oxyhemoglobin in the blood do not release oxygen as readily as is the case with normal blood. Toxic reactions following inhalation of carbon monoxide and the conversions of hemoglobin to carboxyhemoglobin are primarily the result of tissue anoxia which can cause tissue changes. For this reason, prolonged breathing of carbon monoxide, even in very low concentration, can cause permanent damage especially to the heart and central nervous system. Administration of pure oxygen relieves the immediate ill effects since oxygen greatly increases the conversion of carboxyhemoglobin to the oxyhemoglobin form. The presence of carboxyhemoglobin can be recognized by the brilliant cherry-red color of the blood specimen.

Physiologic hemoglobins can also be converted to methemoglobin, or ferrohemoglobin. In methemoglobin, the ferrous iron (Fe^{+2}) in the heme complex has been oxidized to the ferric form (Fe^{+3}). Methemoglobin is a part of the "inactive" hemoglobin in the body and is unable to combine reversibly with oxygen and carbon dioxide. This inability, for obvious reasons, hinders the transfer of oxygen from the blood to the tissues, and abnormal amounts of methemoglobin result in a functional "anemia" with cyanosis. It should be noted that in this condition there may not be any decrease in the number of red blood cells or in the concentration of hemoglobin because the defect is in the oxygen-carrying capacity of the molecule. Small amounts of methemoglobin are present in normal blood due to oxidation of hemoglobin by peroxide. It is believed that 99.6 percent of the iron in hemoglobin exists as ferrous molecules with about 0.4 percent in the ferric or oxidized state. The small amounts always being formed are reduced to the physiologic form by the action of red blood cell enzyme systems and by reducing agents, such as glutathione and ascorbic acid, found in blood cells. In the normal individual, the concentration of methemoglobin seldom exceeds 0.06 grams per deciliter of blood. In the abnormal individual, this value may be much increased, and treatment with ascorbic acid (vitamin C) or methylene blue may reduce ferric iron to ferrous iron. Methemoglobinemic blood has a characteristic brown color. Some individuals inherit genes that are deficient in coding for the normal enzyme systems that reduce the normally small amounts of methemoglobin to usable hemoglobin; thus, their methemoglobin levels rise. Others acquire methemoglobinemia by ingesting certain chemicals that contain nitrites or nitrates, such as nitroglycerin and amyl nitrate.

Another form of hemoglobin that may occur abnormally is sulfhemoglobin. It may be present simultaneously with methemoglobin. In sulfhemoglobin, sulfur that has been ingested in the form of sulfonamides, phenacetin (as in APC tablets), and even Bromo-Seltzer (in large quantities) becomes attached to the globin portion of hemoglobin, rendering the molecule useless. The reaction is not reversible in the body, and the dark red sulfhemoglobin remains as long as the red cells in which it resides, survive.

A well-informed, trained technician can accurately quantitate hemoglobin levels in a patient's blood. If the procedure is inherently accurate and is properly performed, the final report offers the physician one of the simplest means of detecting the existence and degree of anemia and of evaluating the results of treatment of this condition. Many methods of determining hemoglobin concentration have been used over the years in the quest of a single test which is accurate, simple, and reproducible. These methods, depending on the technique employed, can be divided roughly into four groups: gravimetric, colorimetric, gasometric, and chemical. Methods in the latter two groups are, as a rule, too complicated for general use in a clinical laboratory, so we will discuss only gravimetric and colorimetric methods.

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In the gravimetric procedure, the specific gravity of the blood in question is determined by allowing a drop to fall into each of a series of copper sulfate solutions of known specific gravity. Immediately on contact with the copper sulfate the blood becomes encased in a sack of copper proteinate. As a result, the blood remains as a discrete drop without dispersal or specific gravity change for a period of fifteen to twenty seconds. The drop of blood is observed after it has reached equilibrium. We note if it sinks, remains stationary, or rises--in other words to see if it has a higher, the same, or lower specific gravity than the copper sulfate solution. By this method, the specific gravity of blood can be determined with an accuracy of plus or minus 0.001. The specific gravity figure is then converted to grams of hemoglobin per deciliter of blood, using a prepared nomogram or graph. This method is primarily used as a screening procedure for blood donors, where the minimum acceptable limits of hemoglobin concentration are 12.5 grams per deciliter for females (specific gravity 1.053), and 13.5 grams per deciliter for males (specific gravity 1.055). Only two cylinders of copper sulfate are required for this screening, corresponding to the two specific gravity minima for each sex. Blood is collected by capillary puncture from the prospective patient and dropped into the appropriate cylinder. Up to a total of one test per milliliter of copper sulfate may be run in the same vial, though only about 75 percent of this amount is recommended. There are several other advantages: the solutions are nontoxic; their coefficient of expansion at ambient temperatures is essentially that of blood so that a minimum of temperature regulation is required; and the dispersal of blood in these solutions is slow enough for accurate observations to be made. The major disadvantage of this procedure lies in the fact that it is based on the assumption, admittedly inaccurate, that hemoglobin concentration is directly proportional to blood specific gravity. However, in spite of this drawback, the gravimetric, specific gravity, or copper sulfate method remains effective as a mass screening device for blood donors.

Colorimetric procedures are so designated because the final determination of hemoglobin concentration is made after the comparison, in some way, of the color of the unknown specimens with the color of a standard. These colorimetric procedures can be divided into two classes, visual and photometric (or spectrophotometric), depending on whether the color comparison between the unknown and the standard is made by the eye or by an instrument. Each of these two classes can be further divided into direct methods, where hemoglobin is determined as unaltered hemoglobin, and indirect methods, in which hemoglobin is converted to some other compound before the color comparison is made. In general, the direct visual methods cannot be recommended for use because of the excessive error inherent in any attempt to match colors by the human eye. An example of the direct visual procedure is the method of Tallqvist, in which undiluted blood from capillary puncture was collected on a small sheet of absorbent paper and the color compared to a standard lithographed color scale. The error inherent in this test was in the range of 20 to 50 percent. Another direct visual method was that of Dare. In this procedure, untreated whole blood was drawn by capillary

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action between two glass plates and the color matched against a rotating disc of tinted glass which varied in thickness and depth of red color. The Dare hemoglobinometer was expensive and, though the results were more accurate than the Tallqvist's method, errors of twenty to thirty percent have been reported. The A.O. Spencer Hemoglobin Meter is a step-up in sophistication of the visual direct application. Here, a blood sample is placed on a special slide, the cells lysed with an agent known as saponin, and then displayed in a special reading device. Again, the color comparison is made by the human eye, but in this method, cells are not present and the conditions are more controlled. Because of the inaccuracy in comparison, these direct visual methods are not used in modern clinical laboratories. (The A.O. Spencer Hemoglobin Meter is used in some field kits by Special Forces personnel.)

Although not completely satisfactory, the Sahli-Hellige hemoglobin determination procedure, a visual indirect method, is more accurate than the colorimetric methods we have already mentioned. This procedure is based on conversion of the hemoglobin in a measured amount of blood to acid hematin by the action of dilute hydrochloric acid. The yellowish-brown acid hematin is diluted with distilled water, drop by drop, until a visual color match against a permanent colored glass standard is obtained. This conversion and dilution is accomplished in a square glass tube which has two scales etched on its sides, one reading in percent and the other allowing a direct reading in grams per deciliter of blood. Only the use of the latter scale gives acceptably accurate and meaningful results. Color development is progressive, rather than instantaneous; maximum intensity is reached after one hour. However, increase in color intensity is insignificant after forty minutes, and ninety-five percent of the final intensity is attained in ten minutes. For this reason, readings may be made after ten minutes, but the instrument must be standardized for the prescribed time interval which must be adhered to with each procedure. A disadvantage of this method lies in the addition of the water. This is a fairly delicate step, since the entire procedure must be repeated if one or two excess drops of water are added and the point of color match is passed. The personal factor introduces error of varying magnitudes, as it is very difficult to match colors exactly by the human eye. On the other hand, Sahli's method of hemoglobinometry is fairly simple in principle and performance, requires no expensive equipment, and uses readily available reagents. Carefully monitored, the Sahli procedure gives clinically significant results acceptable for use in a field medical unit.

Photometric methods of hemoglobinometry where color match of the unknown with the standard is obtained by an instrument are more accurate than methods requiring a visual color match because the human factor is eliminated. The first photometric hemoglobin determination procedure of Sheard and Sanford used 0.1 percent sodium carbonate as a diluent to hemolyze the red blood cells and release the hemoglobin. This photometric procedure is considered a direct method because the hemoglobin is not converted to another compound but is compared photoelectrically as oxyhemoglobin against known standards. The color of the oxyhemoglobin solutions tends to fade rapidly; and, consequently, values vary according to time of reading after dilution of both standard and unknown.

Though this direct photometric method represented an advance in hemoglobinometry, the unstable color of oxyhemoglobin is definitely a disadvantage, even with the substitution of ammonium hydroxide for sodium carbonate as the hemolytic reatent. However, it can be stated that this method yields a very useful figure: the amount of hemoglobin capable of carrying oxygen as opposed to the total amount of hemoglobin, whether or not in active form.

Nevertheless, continuing efforts were made to find a more accurate and reproducible method of determining hemoglobin concentration; a method in which the final color was stable and the time of color comparison was not a factor. In the United States, these efforts culminated in the standardization of an indirect photometric procedure recommended for hospitals by the National Academy of Sciences, National Research Council. This was the cyanmethemoglobin method.

The test is based upon the conversion, by a reagent, of all types of hemoglobin in the patient's blood (with the exception of sulfhemoglobin) to cyanmethemoglobin, a compound found stable up to six years in the refrigerator. The concentration of cyanmethemoglobin in solution is proportional to the amount of hemoglobin originally present in the patient's blood. When the solution is placed between a light source of certain wavelength and a photoelectric cell, the solution absorbs a portion of the light, proportional to the amount of cyanmethemoglobin present. This causes the photoelectric cell to pick up and register less light than if no blood had been present in the reagent (Beer's Law). Therefore, since some of the incident light was absorbed by the cyanmethemoglobin solution, only a fraction of the incident light was transmitted. This fraction may be expressed on the spectrophotometer as percent transmittance. This, in turn, may be converted to grams of hemoglobin per deciliter of blood by comparing it to the percent transmittance of a known standard concentration. The standard is a manufactured product, dilutions of which may be transposed to a graph or chart for routine use.

For the cyanmethemoglobin method, an aspirator is required to draw the patient's blood sample into the Sahli pipet. This pipet has no mixing bulb and is calibrated to contain twenty cubic millimeters, equal to twenty lambda, or 0.02 milliliters of blood. In addition, several 13 x 100 mm test tubes and several 19 x 150 mm cuvets will be needed. A spectrophotometer, warmed up, calibrated, and in good working order is also necessary.

The reagent used in this test is a cyanide compound solution named after Drabkin, who initiated the procedure. Drabkin's solution contains sodium bicarbonate, potassium ferricyanide, and potassium cyanide. Exercise care in handling this reagent. The sodium bicarbonate lyses or breaks

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open the red blood cells. Potassium ferricyanide then converts the hemoglobin in the blood to methemoglobin by oxidizing the iron of the hemoglobin from the ferrous to the ferric form. The methemoglobin reacts with potassium cyanide to form cyanmethemoglobin, a compound whose color is very stable and whose optical density is directly proportional to the original hemoglobin concentration. Stock standards are available which are accurate within plus or minus two percent of the stated concentration (usually about 60 mg/dl). By obtaining the percent transmittance of at least three known dilutions of this stock standard, a very accurate concentration curve can be prepared. This curve enables the technician to convert the percent transmittance reading of an unknown cyanmethemoglobin solution directly to grams of hemoglobin per deciliter of blood. Because all forms of hemoglobin (except sulfhemoglobin) are converted to cyanmethemoglobin, it may be stated that this is misleading as an indicator of the oxygen-carrying capacity of the blood. Inactive forms of hemoglobin, such as carboxyhemoglobin (which may reach values as high as one and a half grams per deciliter of blood in heavy smokers), methemoglobin, and sulfhemoglobin do not carry oxygen, but yet contribute to the total hemoglobin reading. However, for computing red blood cell indices, the cyanmethemoglobin value is mandatory. Unless a patient has one of the above disorders, this method is also quite acceptable. A competent technician, using a properly adjusted spectrophotometer, accurate pipets, properly prepared Drabkin's solution, and a standard curve, can report results with an error of less than five percent and as low as two percent. Because of its accuracy and uniformity, the cyanmethemoglobin procedure has now been accepted as the method of choice for hemoglobinometry in fixed military medical installations of the United States Army.

To perform the cyanmethemoglobin procedure, first mix by inversion the patient's blood specimen, if collected by venipuncture. (A venous specimen yields more reproducible hemoglobin values than a capillary specimen.) Then, pipet blood to the twenty cubic millimeter mark being careful not to overshoot. Wipe the outside of the pipet clean with tissue and deposit it in the bottom of a 13 x 100 mm test tube containing exactly five milliliters of Drabkin's solution. When all the blood has left the pipet, gently raise the pipet until the tip is just under the surface of the Drabkin's solution. Rinse the pipet carefully four times with the relatively clean Drabkin's at the top, insuring that all the blood in the pipet is rinsed out into the solution. If the pipet is not rinsed, much of the twenty cubic millimeters of blood will remain in the pipet and the final hemoglobin reading will be misleadingly low. When this step is completed, thrust the pipet tip back down to the bottom of the test tube and blow vigorously to mix the blood with Drabkin's. Set the labeled test aside for ten minutes to allow the reaction to go to completion. The blood has now been diluted one to 251 (0.02 ml in total volume of 5.02 ml of solution).

Set the wavelength of light on the spectrophotometer to 540 nanometers; at this wavelength the light is green which is the color that is primarily

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absorbed by cyanmethemoglobin and therefore gives the most accurate values. Next, "zero" the instrument which means that when no light is allowed to pass through the cuvet well of the instrument, the spectrophotometer will register zero percent light transmitted. To accomplish this, insure that the light in the cuvet well is blocked off with the rotating sleeve or holder. Slide the scale on the spectrophotometer so that the hairline indicator of the galvanometer reads exactly zero. Then, "blank" the machine with plain Drabkin's solution. The Drabkin's is placed into a clean, dry, unscratched cuvet which is then inserted into the cuvet holder in the cuvet well. The holder is turned ninety degrees to allow the green light to pass through the specimen and reach the photocell. Adjust the galvanometer settings (coarse and fine knobs on most instruments) so that the galvanometer reads precisely 100 percent transmittance. Do not touch the sliding plastic scale that was moved for the zeroing procedure. Use only the indicated knobs to set the galvanometer. The purpose of this procedure is essentially to subtract the quantity of light absorbed by Drabkin's solution alone from the subsequent reading of the blood plus Drabkin's, so that the amount of light absorbed when the specimen (unknown) is read will be due only to the concentration of the hemoglobin. One further point about using cuvets: not only must they be wiped clean of fingerprints and dirt before inserting them into the instrument, but they must be aligned properly once the light is passing through them. Most manufacturers place their trademark on the side of the cuvet which is most optically perfect so that the technician will know to turn that side facing the light beam. This should be done each time the light is passed through a cuvet. The cover should be placed over the well to eliminate exterior light.

Finally, the specimen may be read for its hemoglobin content. Transfer the patient's specimen to a cuvet without introducing any bubbles that would scatter light. After wiping and aligning the cuvet in the well, place the cover over the well and read the percent transmittance (the bottom scale on Coleman models) for the specimen. Locate this value on the conversion chart made from the certified standards and convert to grams of hemoglobin per deciliter of blood. Only this value is reported to the physician. When the spectrophotometer readings are completed, ensure that the light source is blocked off from the photocell and the instrument is left on and unmoved.

An important source of error that can be avoided by good general laboratory supervision is the calibration and stability of the spectrophotometer. The more frequently the instrument is moved and turned on and off, the less accurate are the readings. It should be checked periodically with a didymium filter and fresh standard solution. Similarly, the Drabkin's solution should be changed weekly since it is not stable and deteriorates.

The individual technician must attend to his own province of responsibility in performing pipeting, wiping, rinsing, and reading the

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spectrophotometer properly. Failure to perform any of these operations meticulously results in serious inaccuracies. Inspect cuvets for scratches because they may deflect a significant amount of light. If dry cuvets are not available, cuvets rinsed with deionized water may be used, provided that all cuvets are wet but shaken as dry as possible before use.

The range of normal hemoglobin values varies significantly with age and sex. Blood of newborn infants may contain from 18 to 27 grams per deciliter of blood; in children aged six to twelve, the levels generally fall to 10 to 15 grams per deciliter of blood. As the child matures, the hemoglobin again rises to a range of 14 to 17 grams per deciliter of blood in the adult male, while the adult female's blood usually contains 12 to 16 grams per deciliter of blood. No sharp line of demarcation separates normal from abnormal values, and any single report of hemoglobin concentration on an individual will be interpreted by the physician in the light of age, sex, and total clinical picture.

Anemia is a general term that covers several classes of disorders in which the patient's red blood cells are deficient in quantity and/or quality. Therefore, in many anemias the hemoglobin concentration reflects the deficiency by being decreased below the normal value. There are many different kinds and causes of anemia, some of which have already been discussed. Suffice it to say, for the purposes of a basic medical laboratory course, there are many other causes that will affect the patient's hematologic picture.

Patients with polycythemia vera and patients badly burned are two cases in which the red blood cell count are often increased. Consequently, the hemoglobin concentration may reflect these increased numbers of cells. Polycythemia is the result of over-production of red cells, while in the burn patient the case is one of loss of fluid from the plasma, resulting in an increased concentration of red blood cells.

Without hemoglobin and its strong affinity for oxygen, the human body would require at least 100 times the amount of blood available to maintain itself. In addition to this vital function, molecules of hemoglobin, present in the millions in each red blood cell, are important in acid-base balance and carbon dioxide removal. Therefore, it is obvious why a hemoglobin determination done on blood obtained either by a simple capillary puncture or venipuncture is of prime importance to a physician in diagnosing anemias and other illnesses. The laboratory technician must perform with skill and accuracy because his results may profoundly affect the welfare of the patient.

SFC Andre U. Wynohrad

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY LP 08-280-009
LABORATORY SCIENCES DIVISION _ 086
CLINICAL MICROSCOPY BRANCH

Leukocyte Identification and Staining (4 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES:

TM 8-227-4.
Miale, John B., Laboratory Medicine Hematology, 3rd ed. St. Louis, C. V. Mosby, 1967.
Weisz, Paul B., The Science of Zoology, New York, McGraw Hill, 1966.
Wintrobe, Maxwell M., Clinical Hematology, 6th ed. Philadelphia, Lea and Febiger, 1967.

STUDY ASSIGNMENT: After class study TM 8-227-4, pp 3-8 to 3-12, 4-5 to 4-14, and 5-25 to 5-28.

STUDENT UNIFORM AND EQUIPMENT: None

TOOLS, EQUIPMENT, MATERIALS: None

PERSONNEL: One primary instructor (MOS 92B4H, 92B3H or 92B2H)

INSTRUCTIONAL AIDS: PA system with lapel microphone, chalk, chalkboard and eraser

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

I. INTRODUCTION: (5 min)

A. Opening Statement: The differential count may be the single most important diagnostic tool available to the physician. The "WBC" count is an index of leukocytosis or leukopenia and may be useful in determining the general condition of the patient, but it is the differential count that is most helpful in determining what pathological condition(s) may be present in the patient. In fact, the differential is so important to the diagnosis, that it is requested on a "routine" basis. However, there is

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*This lesson plan supersedes LP 08-280-009, 075.

no such thing as a "routine" differential to the laboratory technician. The technician who allows the differential to become "routine" will miss important morphology that is necessary to the physician in order for him to correctly diagnose and treat the patient.

B. Objectives

1. State the characteristics of a thin blood smear suitable for microscopic examination.

2. Explain the procedure for performing the Wright's staining technique, and name the components of the stain and the sources of error encountered.

3. Outline the procedure for preparing and staining a thin blood smear for microscopic examination.

4. Identify the various normal leukocytes and list their characteristics, and state the criteria used in their identification.

5. Discuss the purposes and principles of the differential count, and cite both normal and abnormal conditions encountered.

C. Class Procedure and Lesson Tie-in

1. First four hours of a block of instruction on identification of peripheral blood leukocytes and the principles of blood film preparation and staining.

2. Practical exercise in accordance with individual needs.

3. Conference when necessary to solve individual problems.

II. EXPLANATION (165 min)

A. Thin Blood Smear Preparation

1. Principle

QUESTION: Why do we wish to prepare a thin blood smear?

ANSWER: A thin blood smear is prepared so that the blood cells will be on the slide in a single cell thickness for staining, microscopic examination and differential cell counting.

2. Equipment

   a. Clean glass slides

   b. Capillary tubes (or venipuncture or cipillary puncture equipment)

3. Procedure

   a. Place drop of blood on slide (1/8" to 3/16" diameter)

   b. Place slide on flat surface and hold firmly

   c. Pull "spreader" slide back at 30° angle

   d. Allow drop to flow toward edges of slide

   e. Push "spreader" slide forward with smooth, rapid motion (pulling cells into the smear pattern)

   f. Allow slide to air dry (do not blow on smear)

   g. Write patient's name in thick portion (with pencil)

4. Characteristic of suitable smear

   a. Length (1/2 to 3/4 length of slide)

   b. Width (margin on both sides of slide)

   c. Quality

      (1) Thick portion (rouleaux formation)

      (2) Thin portion (even distribution of leukocytes)

      (3) Feathered edge (aggregations of larger leukocytes)

5. Sources of error

   a. Short smear (reduce angle of "spreader" slide)

   b. Long smear (increase angle of "spreader" slide)

   c. No margins (smear before blood flows to edges of slide)

   d. No feathered edge (continue smearing entire length of slide)

B. Staining Blood Smears

   1. Principle

QUESTION: Why do we stain the cells on a blood smear?

ANSWER: The cells are almost colorless in appearance; therefore, we wish to dye them so that the various structures within the cell will be identifiable.

2. Reagents
    a. Wright's stain (polychromatic stain)
        (1) Methyl alcohol (dehydrates and fixes cells)
        (2) Eosin (red stain - dyes basic portion of cell)
        (3) Methylene blue (blue stain - dyes acid portion of cell)
    b. Wright's buffer
        (1) Phosphate buffer (activates staining mechanism)
        (2) pH range (critical: pH 6.4 to pH 6.8)
3. Procedure
    a. Flood slide with stain (2 to 4 minutes)
    b. Add equal volume of buffer (2 to 6 minutes)
    c. Mix by blowing, and allow to stand
    d. Wash with distilled water
    e. Allow to air dry
4. Sources of error
    a. Alkaline stain (over-all blue color - increase buffer time)
    b. Acid stain (over-all red color; pale leukocytes - decrease buffer time)
    c. Precipitate
        (1) Filter stain daily
        (2) Wash slide for extended time with water

C. Differential Cell Count

  1. Purposes

    a. Identify types of leukocytes

    b. Report percent occurrence of leukocytes (aid in diagnosis)

  2. Equipment

    a. Microscope (with light source)

    b. Differential cell counter

    c. Immersion oil

  3. Procedure

    a. Low power (locate thin portion of smear and gain overall impression of slide)

    b. Oil immersion (examine smear: counting 100 leukocytes with prescribed "criss-cross" pattern)

  4. Sources of error

    a. Thick portion

      (1) Leukocytes not flat (not able to see cellular structures clearly)

      (2) Uneven distribution of cells (increase of small cells)

    b. Feathered edge (increase of larger cells)

    c. Improper objective (unable to see intracellular structures and abnormalities)

  5. Unit of report (percent of each type of cell seen)

D. Review of Cellular Biology

  1. Cell ("atomic" units of living matter)

  2. Origin (cells arise only from living cells)

  3. Structure

- a. Nucleus
    - (1) Nuclear membrane
    - (2) Nucleoplasm (gel-like nuclear sap)
    - (3) Chromatin material (chromosome with DNA and RNA)
    - (4) Nucleolus ("little nucleus," spherical body with RNA)
- b. Cytoplasm
    - (1) Cell membrane
    - (2) Organelles (mictochondria, ribosomes, Golgi bodies, and centrioles)
    - (3) Granules
    - (4) Vacuoles

E. Criteria for Identification of Leukocytes

1. Size and shape

NOTE: Erythrocytes are used as a scale with standard size of 7.0 microns.

- a. Overall size of cell (in microns)
- b. Overall shape of cell

2. Cytoplasm

- a. Amount (compared to quantity of nucleus)
- b. Color

3. Nucleus

- a. Shape
- b. Morphology (form and structure of chromatin material)

4. Granules

- a. Size
- b. Color
- c. Distribution

F. Classification of Leukocytes

1. Agranular (no specific granules)

a. Lymphocytes

(1) Size and shape

(a) Size (small - 7 to 9 microns)

(b) Size (large - 10 to 18 microns)

(c) Shape (generally irregular)

(2) Cytoplasm

(a) Amount (usually scanty - but sometimes abundant)

(b) Color (translucent and sky-blue)

(3) Nucleus

(a) Shape (rounded)

(b) Morphology (dense chromatin pattern)

(4) Granules ("azurophilic" when present)

(a) Size (varies)

(b) Color (purple to blue)

(c) Distribution (uneven, with aggregates)

(5) Formation (lymphoid tissue)

(6) Normal value (20 to 35 percent)

(7) Increase (in viral infections)

b. Monocytes

(1) Size and shape

(a) Size (12 to 16 microns)

(b) Shape (irregularly rounded)

(2) Cytoplasm

(a) Amount (abundant)

(b) Color (opaque, blue-gray with "ground-glass" appearance)

        - (c) Vacuoles (considered normal when seen)
    - (3) Nucleus
        - (a) Shape (irregular)
        - (b) Morphology ("lacy" or "spongy" chromatin material)
    - (4) Granules ("azurophilic" when present)
        - (a) Size (varies)
        - (b) Color (purple to blue)
        - (c) Distribution (uneven)
    - (5) Formation (reticuloendothelial system)
    - (6) Normal values (2 to 6 percent)
    - (7) Increased (in chronic infections)

2. Granular (myelocytes with specific granules)
    - a. Eosinophil
        - (1) Size and shape
            - (a) Size (10 to 15 microns)
            - (b) Shape (generally rounded)
        - (2) Cytoplasm
            - (a) Amount (abundant)
            - (b) Color (bluish-pink to colorless)
        - (3) Nucleus
            - (a) Shape (usually bilobed)
            - (b) Morphology (dense, irregular chromatin pattern)
        - (4) Granules
            - (a) Size (large, may appear hollow)

- (b) Color (bright red-orange)
- (c) Distribution (even)

(5) Formation (bone marrow)

(6) Normal value (2 to 4 percent)

(7) Increased (in allergies and some parasitic infestations)

b. Basophil

- (1) Size and shape
  - (a) Size (10 to 15 microns)
  - (b) Shape (generally rounded)
- (2) Cytoplasm
  - (a) Amount (abundant)
  - (b) Color (faint pink to colorless)
- (3) Nucleus (usually obscured)
- (4) Granules
  - (a) Size (large, coarse "chunk of coal" appearance)
  - (b) Color (blue-black)
  - (c) Distribution (uneven - overlay nucleus)
- (5) Formation (bone marrow)
- (6) Normal value (0 to 1 percent)
- (7) Increased (in leukemias)

c. Neutrophils (segmented neutrophil, "seg" and "poly")

- (1) Size and shape
  - (a) Size (10 to 15 microns)
  - (b) Shape (generally rounded)

(2) Cytoplasm

(a) Amount (abundant)

(b) Color (pink)

(3) Nucleus

(a) Shape (2 to 5 lobes or segments)

(b) Morphology (dense, irregular chromatin pattern)

(4) Granules

(a) Size (very small, uniform "pinpoints")

(b) Color (pink to light purple)

(c) Distribution (evenly distributed)

(5) Formation (bone marrow)

(6) Normal value (55 to 65 percent)

(7) Increased (in bacterial infections)

d. Bands (banded neutrophil)

(1) Size and shape

(a) Size (10 to 16 microns)

(b) Shape (generally rounded)

(2) Cytoplasm

(a) Amount (abundant)

(b) Color (pink)

(3) Nucleus

(a) Shape ("sausage" or "horse shoe" shaped)

(b) Morphology (compacted, irregular chromatin pattern; lobes not pinched or segmented)

- (4) Granules
    - (a) Size (very small, uniform "pinpoints")
    - (b) Color (pink to light purple)
    - (c) Distribution (even)
- (5) Formation (bone marrow)
- (6) Normal value (3 to 5 percent)
- (7) Increased (in bacterial infections)

G. Leukocytic Abnormalities

- 1. Atypical lymphocytes (virocytes)
    - a. Occurrence (viral diseases)
    - b. Appearance
        - (1) Size (up to 20 microns)
        - (2) Cytoplasm (basophilic and foamy or patchy with occasional vacuole)
    - c. Report (occurrence as percentage of lymphocytes)
- 2. Hypersegmented neutrophils
    - a. Occurrence (pernicious anemia)
    - b. Appearance (6 or more lobes)
    - c. Report (occurrence, but not number)
- 3. Pyknotic neutrophils
    - a. Occurrence (older neutrophils)
    - b. Appearance (dense nuclei; not connected or lobed)
- 4. Toxic granulation
    - a. Occurrence (toxic condition in neutrophilic series)
    - b. Appearance (coarse dark purple granules)
    - c. Report (occurrence, but not numbers)

5. Shilling's shift (physician's use: indication of prognosis)

   a. Shift-to-left (immature forms - regenerative shift)

   b. Shift-to-right (mature forms - degenerative shift)

H. Questions From Students

III. SUMMARY (10 min)

A. Review of Main Points

1. Preparation of thin blood smears

2. Staining

3. Differential cell count

4. Criteria for identification

5. Classification of leukocytes

B. Closing Statement: The skill you develop in making, staining and reading blood smears will depend upon how well you apply the techniques and principles you have learned today. The accuracy of the differential count is based on the quality of the preparation and staining of the blood film, as well as your skill in identifying the various cells. Remember, you are the eyes of the physician (in the laboratory) and what you see and report will guide him in the diagnosis and treatment of the patient.

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*IM 08-280-009
086

Leukocyte Identification

The differential count may be the single most important diagnostic tool available to the physician. The "WBC" count is an index of leukocytosis or leukopenia and may be useful in determining the general condition of the patient, but it is the differential count that is the most helpful in determining what pathological conditions(s) may be present in the patient. In fact, the differential is so important to the diagnosis, that it is requested on a "routine" basis. However, there is no such thing as a "routine" differential to the laboratory technician. The technician who allows the differential to become "routine" will miss important morphology that is necessary to the physician in order for him to correctly diagnose and treat the patient.

If we take a drop of blood and just place it on a slide, and then look at it under the microscope, we immediately see that any systematic examination of the blood would be impossible. The cells lack any definition of clear color, and the thickness of the drop prevents us from seeing the individual cells clearly. Therefore, it is necessary to smear out the drop of blood so that the blood cells will be on the slide in a single layer thickness for staining, microscopic examination and differential counting.

To prepare the thin blood smear, we need clean glass slides and capillary tubes. The slides must be scrupulously clean, since any dirt, grease or oil will result in defects (or holes) in the smear, interfering with microscopic examination. The capillary tubes are used in transferring blood to the slides. (However, blood for the smear may be obtained directly from the venipuncture needle, or from the site of capillary puncture, if the smears are prepared at the time the blood specimen is collected.)

Thin blood smears are prepared as follows:

1. Place a small drop of blood on a clean glass slide. The size of the drop will vary from one individual to the next; however, a drop of blood about 1/8" to 3/16" in diameter is generally satisfactory for most technicians.

2. Place the slide on a flat surface and hold it firmly by the edges. This will prevent the slide from moving while preparing the smear.

3. Pull the "spreader" slide back at an angle of approximately 30 degrees from horizontal.

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*This instructor's manuscript supersedes IM 08-280-009, 075.

4. Allow the "spreader" slide to come into contact with the drop and allow blood to flow toward the edges of the slide.

5. Before the blood reaches the edges of the slide, the smear is made by pushing the "spreader" slide foward with a smooth, rapid motion. This action pulls the cells in the drop of blood into the smear configuration.

6. Allow the smear to air dry. Do not blow on the smear; moisture from your breath will cause crenation of the erythrocytes, obscuring any morphology.

7. The slide must be clearly labeled to positively identify the specimen. Labeling is most easily accomplished by writing the patient's name in the thick portion of the smear with a lead pencil. Thin blood smears suitable for microscopic examination should be from 1/2 to 3/4 of the length of the glass slide. Blood smears should have a clearly defined margin on each side of the glass slide. The presence of these features will help to insure that we have a slide suitable for microscopic examination. The smear should include three distinct areas: (1) thick portion, where cells may be observed in rouleaux formation; (2) thin portion, where we will find the erythrocytes evenly distributed (that is, just touching one another), and where we will find an even distribution of leukocytes; and (3) feathered edge, where we will find large spaces between the erythrocytes, and aggregations of the larger leukocytes.

It is necessary to stain the smear before microscopic examination, because the unstained cells appear almost colorless when viewed under the microscope. Wright's stain is used to dye the various structures within the cell, thereby making examination and study of the intracellular structures possible.

Wright's stain contains methyl alcohol, which serves as the vehicle in which the analine dyes are dissolved. The methyl alcohol acts to dehydrate and fix the erythrocytes and leukocytes and bonds them to the glass of the slide. Eosin is a red-orange stain, which dyes the basic portions of the cell. The acid portions of the cell are dyed blue by the action of methylene blue.

Wright's buffer is a phosphate buffering solution which activates the staining mechanism of the Wright's stain. Most of the staining actually takes place only after the buffer has been applied. It should be noted that the buffer is slightly acid with a pH range between pH 6.4 and pH 6.8. It is not possible to obtain a suitable stain unless the pH of the buffer falls within this range.

The technique for performing the Wright's stain is as follows:

1. Flood the smear with a small amount of Wright's stain. A laboratory wash bottle or eye dropper may be used to facilitate handling the stain. Allow the stain to stand for the specified "stain time." (NOTE: "stain time" will vary from two to four minutes.)

2. Add an equal volume of Wright's buffer.

3. Mix the stain and buffer by blowing on the slide until a metallic sheen is formed on the surface of the mixture. Allow the mixture to stand for the "buffer time." (NOTE: "Buffer time" will vary from two to six minutes, depending on the individual batches of stain and buffer. "Stain time" and "buffer time" must be adjusted by "trial-and error" until suitable staining is achieved.)

4. Wash with distilled water to remove the staining solutions. The residue of stain may be removed from the back and edges of the slide with a dry gauze sponge. Care must be taken not to touch the smear, as it will be damaged or destroyed by contact with the sponge.

5. Allow the slide to stand on edge and air dry.

The stained smear should have a balance of reds and blues, with the erythrocytes having a buff color. Adjustments of the staining and buffer times can prove frustrating at times, but it is easier if we remember that the buffer is just slightly acid. If the stain is alkaline (that is, the slide is bluish in overall appearance), the buffer time may be increased slightly to give the correct color balance. Buffer time is decreased to compensate for an acid stain (overall red color with pale staining leukocytes).

Precipitation from the Wright's stain may be eliminated or reduced by filtering the staining solution daily. However, if precipitate is present, it may usually be reduced or eliminated by washing the stained slide under running water for a short period of time.

The differential count is an invaluable aid to the physician in reaching a diagnosis and in following the patient's response to therapy. When performing the differential count, we identify the types of leukocytes present and report the percentage occurrence of each type of leukocyte to the physician.

The differential count should always be initiated utilizing the low power objective (10x) and rapidly scanning the slide to gain an overall impression of the condition of the smear. Then we should change to the high power objective (43x) and locate the thin portion of the smear for further examination. (NOTE: This step is optional. The thin portion of the smear may usually be located under low power.) The actual differential count is performed with the oil immersion objective (97x). To use the immersion objective it is necessary to place a drop of immersion oil on the slide so that the oil immersion lens is actually touching the oil.

The differential count is performed by counting 100 leukocytes. The standard "criss-cross" pattern is utilized. The smear is examined systematically from one side to the other; the smear is moved toward the thick portion of the smear approximately two oil immersion fields, and traversed to the opposite side of the smear; and so on until 100 leukocytes have been located and identified. The results of the count are then reported by listing the occurrence of each type of cell seen per 100 leukocytes (percent), on the report form.

Care must be exercised to insure that leukocytes are located and identified in only the thin portion of the smear. Identification of cells in the thick portion of the slide is difficult; because of the thickness of the slide the cells tend to obscure one another. The leukocytes cannot flatten out; therefore, intracellular structures are obscured, further complicating identification. There is also a disproportionate increase of smaller cells in the thick portion of the smear, and aggregates of larger nucleated cells in the feathered edge. The anticipated distribution of nucleated cells is found only in the thin portion of the smear.

A common error in performing the differential count is using a less powerful objective than the oil immersion objective, which is designed to give maximum magnification and resolution to the field of view. The more experienced technician recognizes the importance of studying the intracellular structures when identifying the various leukocytes. When using the high power or low power objectives we are not able to clearly see the intracellular structures and/or abnormalities.

By the way of review, we recall that all living things are composed of cells. The simplest unicellular organism (e.g., bacteria) to the most complex plants (e.g., trees) and animals (e.g., humans) are composed of cells. All life forms, as we know them, are constructed of these "atomic" units of living matter which arise only from similar living cells.

Animal cells are characterized by subdivision of the cells into a nucleus which is sourrounded by the cytoplasm. The nucleus is bounded by the nuclear membrane and is composed of the gel-like nuclear sap, or nucleoplasm in which the chromosomes or chromatin material is suspended. The chromosomes are composed of the nucleoporteins DNA and RNA; they are usually well-defined only at the time of cellular reproduction. The nucleolus ("little nucleus"), which may or may not be visible, is associated with cell division.

The cytoplasm consists of a semi-fluid ground substance, which surrounds the nucleus, and is contained within the dark staining cell membrane. There are several organelles in the cytoplasm, which include mitochondria (involved with energy producing reactions), ribosomes (enzyme synthesis), Golgi bodies (secretion), and centrioles (cell reproduction). Apart from the organelles just listed, animal cytoplasm generally contains additional granules and/or vacuoles. They may be vehicles transporting raw materials from the cell surface to its interior, synthesized products in the opposite direction, or they may be places of storage, or special processing centers. Granules are prominent in most human leukocytes. Vacuoles are infrequently encountered, and considered normal only in the monocytes.

The identification is based on four main criteria: (1) size and shape of the cell, (2) cytoplasm color and its relative quantity as compared to the nucleus, (3) nuclear shape and morphology (the form and structural appearance of the chromatin material), and (4) presence of granules and their size, color and distribution.

Each leukocyte will be described in terms of these four criteria. The leukocytes are grouped into two broad categories: granular and agranular, depending upon their typical cytoplasmic granulation. The cells of the agranular series will be discussed first. The agranular leukocytes may contain small non-specific granules; this series includes the monocyte and the lymphocyte.

Lymphocytes may be classed in two distinct size groups or "races." The "small race" lymphocyte measures from **seven to nine microns in diameter,** while the "large race" lymphocytes measure from 10 to 18 microns in diameter. Aside from this size difference there are few morphological variations, and they should be enumerated without regard for size. The lymphocyte is generally rounded in shape, but may appear oval or even spindle shaped on occasion. The cytoplasm is translucent and sky-blue. The cytoplasm is usually scanty (particularly in the "small race" lymphocyte) but may be abundant. The eccentrically located nucleus is large and rounded with a dense chromatin network. Non-specific azurophilic granules are usually present in the cytoplasm, varying in size and uneven in their distribution. (These granules differ from those in the granular series in that they fail to give oxidase and peroxidase reactions.) The normal differential count should show 20 to 35 percent lymphocytes. An increased number of lymphocytes is seen in many types of viral infections.

The monocyte is the second type of agranular cell. The monocyte is considered the largest normal cell routinely encountered in the peripheral blood, measuring 12 to 16 microns in diameter. The cytoplasm is usually abundant and has a foamy or "ground glass" appearance which may appear opaque. The nucleus is large and irregular; it may be lobulated, but should not be segmented into distinct lobes. The chromatin strands are not tightly woven, giving the nucleus a loose "spongy" or "lacy" appearance. Vacuoles are frequently observed, and should be considered normal when encountered. The monocytes are formed in the reticuloendothelial system. Two to six monocytes are anticipated in the normal differential count. Monocytosis may be encountered in chronic infections.

The granular or myelocytic series of leukocytes is characterized by specific granulation. The staining characteristics, or the specific granules' reaction to Wright's stain, denote the mature cell's name: eosinophil, basophil and neutrophil.

The eosinophil is characterized by its red-orange granules, as the name implies. The eosinophil is 10 to 15 microns in diameter, and is generally rounded. The cytoplasm is abundant and bluish-pink to colorless when visible. The nucleus is characteristically bilobed, but may be segmented into three or more lobes staining an intense blue. The chromatin material is dense and irregular in appearance. The large red-orange granules may appear to be hollow; they are evenly distributed throughout the cytoplasm, but are not usually superimposed on the nucleus. Eosinophils are formed in the bone marrow. An increase of eosinophils over 2 to 4 percent may be indicative of an allergy or some parasitic infestation.

Large blue-black granules identify the basophil. The average size of this cell ranges from 10 to 15 microns. The abundant cytoplasm is colorless to faint pinkish-blue. The nucleus is usually obscured by the numerous large, coarse blue-black granules which resemble "chunks of coal." On normal peripheral blood smears 0 to 1 percent of the leukocytes may be basophils. Basophils are increased in some types of leukemias.

The neutrophil is 10 to 15 microns in diameter. The abundant cytoplasm is pink and filled with small, uniform "pin-point" granules which stain pink to light purple. The mature nucleus is segmented into from two to five distinct lobes or segments, with a dense, irregular chromatin pattern. Neutrophils are formed in the bone marrow and comprise 55 to 65 percent of the normal differential count. Neutrophilia is present in bacterial infections.

The band is an immature form of neutrophil which is routinely encountered in peripheral blood smears. The band's appearance is identical to the mature neutrophil with the exception of the nucleus. The nucleus is "sausage" or "horse shoe" shaped with nearly parallel sides without pinching into segments or lobes. If pinching has occurred, the cell is considered a mature neutrophil.

(NOTE: At the narrowest point, constriction should be no more than 1/2 the thickness at the broadest point of the nucleus.) The band's chromatin material usually appears less dense than that of the mature neutrophil. Increases of bands over three to five percent are usually encountered in active bacterial infections.

There are several leukocytic abnormalities which must be considered when performing differential counts. While the inexperenced technician may not be expected to positively identify all of these abnormalities and conditions, he should be aware of their existence so that when any abnormalities are encountered he will seek guidance and assistance from a more experienced technician.

Virocytes are encountered in some viral infections, such as those atypical lymphocytes routinely encountered in patients with infectious mononucleosis. Atypical lymphocytes are usually large, up to twenty microns in size. The cytoplasm is usually basophilic, foamy or patchy, and irregular in appearance. Vacuoles are frequently encountered, which aids in the identification of some atypical lymphocytes. The chromatin material is usually coarse and not as evenly distributed nor as dense in the normal lymphocyte. Atypical lymphocytes are significant when encountered in any quantity. The occurrence of atypical lymphocytes is reported as the number of atypical lymphocytes occurring per 100 lymphocytes.

Hypersegmented neutrophils are encountered in patients with pernicious anemia (and folic acid defects), and together with other hematological findings are indicative of this disease. Any neutrophil with six or more lobes or segments is considered to be hypersegmented and should be noted in the morphology section of the hematology report.

Neutrophils past their normal states of development and maturation are said to be pyknotic. The chromatin material continues to become increasingly dense until the lobes of the nucleus completely separate and appear as distinct, separate nuclei. The fragmented nucleus stains an intense deep purple and each fragment may appear to be solid. Pyknotic neutrophils need not be reported, since this is considered a normal (but unusual) stage of cellular development.

Toxic granulation occurs when some toxic substance or condition within the cell interferes with normal cytoplasmic development (e.g., ingestion of heavy metals). Neutrophils are the only leukocytes which exhibit this toxic reaction. Toxic granulation may be observed at any stage of development in the neutrophilic cells. This condition is characterized by appearance of coarse dark purple granules throughout the cytoplasm which are in addition to the anticipated neutrophilic granulation. Toxic granultion may be the first significant indication of some sub-acute toxic conditions, such as accidental ingestion of lead salts, and should always be reported to the physician.

Schilling's shift is not an abnormality in itself, but is an index to the maturity of neutrophilic development. The direction and degree of shift is an indication of the patient's prognosis. A "shift-to-the-left" is characterized by a predominance of immature forms and may be the only indication of infection in the absence of leukocytosis. The presence of immature neutrophils is indicative of a regenerative shift and is usually considered to indicate a hopeful prognosis. The "shift-to-the right" is indicative of a degenerative process and is usually encountered in pernicious anemia and occasionally in chronic morphine poisioning.

The skill you develop in making, staining and reading blood smears will depend upon how well you apply the techniques and principles you have learned today. The accuracy of the differential count is based on the quality of the preparation and staining, as well as the skill you develop in identifying the various cells. Remember, you are the eyes of the physician (in the laboratory) and what you see, will guide him in the diagnosis and treatment of the patient.

SSG Andre Wynohrad
*M 08-280-009
086

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Leukocyte Identification

Cell	Size	Cytoplasm	Nucleus	Granules	Formation	Normal Value	Increased In
Lymphocyte (agranular) small large	 7-9u 10-18u	Transparent, light blue to blue	Large and round	Azurophilic (when present)	Lymphoid tissue	20-35%	Viral infections
Monocyte (agranular)	12-16u	Opaque, gray-blue, ground glass appearance	Loose chromatin, large and irregular	Azurophilic (when present)	Reticuloendothelial system. Spleen, bone marrow, etc.	2-6%	Chronic infections
Neutrophilic Band (granular)	10-16u	Light pink	Sausage or horse-shoe shaped	"pin-point", light pink, evenly distributed	Bone marrow	3-5%	Bacterial infections
Segmented Neutrophil (granular)	10-15u	Light pink	Lobed or segmented	"pin-point", light pink, evenly distributed	Bone marrow	55-65%	Bacterial infections
Eosinophil (granular)	10-15u	Light pink to colorless	Bilobed, segmented	Large, orange red "hollow-spheres"	Bone marrow	2-4%	Allergies & some parasitic infections
Basophil (granular)	10-15u	Light pink to colorless	Usually obscured	Large, vary in size, blue-black	Bone marrow	0-1%	Leukemias

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*This mimeo supersedes M 08-280-009, 014

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CLINICAL MICROSCOPY BRANCH

LP 08-280-014
048

Hematocrit and Sedimentation Rate (2 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic)

PLACE: Classroom

REFERENCES: TM 8-227-4

STUDY ASSIGNMENT: After class study TM 8-227-4, pages 5-14 thru 5-17

STUDENT UNIFORM AND EQUIPMENT: None

TOOLS, EQUIPMENT, AND MATERIALS: None

PERSONNEL: One primary instructor (MOS 92B2H, 92B3H, or 92B4H)

INSTRUCTIONAL AIDS: P.A. system with lapel microphone; chalk, chalkboard, eraser; Videotape #882 and receivers; overhead projector, transparencies

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

I. INTRODUCTION (5 min)

A. Opening Statement: It is interesting to note that the hematocrit, one of the simplest procedures to perform in the hematology laboratory, is also one of the most accurate and valuable procedures. The erythrocyte sedimentation rate, once erroneously thought to be a specific test for pregnancy, is also a simple test to perform.

B. Objectives

1. State the proper steps for performing the microhematocrit and sedimentation rate.

2. Given sources of error select the manner in which a hematocrit or sedimentation rate result would be affected.

3. Select the normal values and proper units of report for microhematocrits and Wintrobe sedimentation rates.

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*This lesson plan supersedes LP 08-280-014,086

C. Class Procedure and Lesson Tie-in

1. The microhematocrit procedure will be demonstrated by means of videotape.

2. This period of instruction will be followed by practical exercise which will include the performance of microhematocrits.

II. EXPLANATION (80 min)

A. Hematocrit

1. Derivation of term (Greek: haimo - blood; krites - to judge)

2. Definition: The volume of erythrocytes in a given volume of whole blood, expressed as the percent by volume of packed red blood cells per deciliter (100 ml) of whole blood.

3. Microhematocrit method

a. Equipment

(1) Capillary tubes

(a) Heparinized

(b) Non-heparinized

(2) Sealing material (clay)

(3) Microhematocrit centrifuge (speed: 10,000 rpm)

(4) Microhematocrit reader

b. Procedure

(1) Fill capillary tubes

(a) Three-fourth full

(b) Two per specimen

(2) Seal dry end (avoid "slanting" of clay plug)

(3) Centrifuge

(a) Place tubes in centrifuge

(b) Hand tighten cover plate

(c) Latch top cover

(d) Set time (5 minutes)

QUESTION: At what speed does the centrifuge operate during the five minute period of centrifugation?

ANSWER: 10,000 rpm

NOTE: Caution should be exercised in using the centrifuge. The top cover should be left in place until the centrifuge has stopped.

(4) Determine results

(a) Align tube in plastic holder

(b) Set red index line to "100"

(c) Intersect plasma-air interface with curved line (move top plate only)

(d) Intersect red cell-plasma interface (rotate both plates)

NOTE: Stress the importance of excluding the "buffy coat" when making the reading.

(e) Read result beneath red index line

c. Normal values and unit of report

(1) Males: 40-54 %VPRC (mean 47 %VPRC)

(2) Females: 38-47 %VPRC (mean 42 %VPRC)

QUESTION: What is meant by the term "anemia"?

ANSWER: A deficiency in the quality and/or quantity of red blood cells.

QUESTION: Will patients with anemia have an increased hematocrit or a decreased hematocrit?

ANSWER: Anemic patients will usually have a decreased hematocrit value.

d. Sources of error

(1) Specimen not adequately mixed

(2) Inadequate volume of specimen used

(3) Improper sealing of tube

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(4) Inadequate/improper centrifugation

(5) Including buffy coat

(6) Improper use of reader

4. Other hematocrit methods

a. Macrohematocrit (Wintrobe) method

(1) Wintrobe tube (larger volume of blood)

(2) Centrifugation (30 minutes at 3,000 rpm)

(3) Normal values (same as for micro-method)

(4) Not commonly employed

b. Electronic cell counters

(1) Hematocrit value computed

(2) Values may differ slightly from manual values

5. Videotape (9 min) (See Annex A)

a. Introduction (1 min)

(1) Microhematocrit Determination (6.07 min)

(2) Points to be observed

(a) Filling tube

(b) Sealing tube

(c) Centrifugation

(d) Reading of results

b. Showing of videotape No. 882

c. Discussion of tape (2 min)

SECOND HOUR

B. Erythrocyte Sedimentation Rate (ESR)

1. Definition: The rate at which red blood cells settle out of whole blood in a given time period, that rate being expressed in mm/hr.

2. Factors affecting the ESR
    - a. Plasma factors
        - (1) Fibrinogen
        - (2) Globulins
    - b. Erythrocyte factors
        - (1) Size of cells
        - (2) Shape of cells
        - (3) Quantity of cells
    - c. Technical factors
        - (1) Temperature
        - (2) Position of tube
        - (3) Air bubbles
        - (4) Delay in performing test
        - (5) Liquid anticoagulants
        - (6) Dirty glassware
        - (7) Disturbance of tube
3. Wintrobe method
    - a. Equipment
        - (1) Wintrobe tubes
        - (2) Rack for tubes
        - (3) Needle (5½ inch) and syringe (or pipet)
        - (4) Timer
    - b. Specimen
        - (1) Venous
        - (2) Anticoagulant (EDTA)

c. Procedure

  (1) Fill tube to "0" mark

  (2) Place in rack

  (3) Set timer (60 minutes)

  (4) Read results

d. Normal values

  (1) Males: 0-9 mm/hr

  (2) Females: 0-20 mm/hr

e. Sources of error

  (1) Inadequate mixing of specimen

  (2) Including buffy coat

  (3) Failure to monitor/control technical factors

QUESTION: Which source of error in performing sedimentation rates is also a source of error in performing hematocrits?

ANSWER: Including the buffy coat when reading the final results.

4. Other ESR methods (e.g., Westergren)

  a. Equipment and procedures similar

  b. Liquid anticoagulants (used in some procedures)

  c. Normal values differ

5. Corrected sedimentation rate (CSR)

  a. Correction for temperature

  b. Correction for hematocrit

NOTE: Many authorities consider corrections of the ESR invalid; however, corrections should be made if so requested by the physician.

6. Significance

  a. Non-specific (suggests disease process or tissue damage)

  b. Not diagnostic (useful in following course of some diseases)

C. Questions from Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Hematocrit

2. Erythrocyte sedimentation rate (ESR)

B. Closing Statement: The simplicity of the hematocrit and sedimentation rate procedures must not lead the technician to believe that errors cannot be made; should he become careless in performing these tests, the physician will be handicapped in his efforts to help the patient.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-045
LABORATORY SCIENCES DIVISION 028
CLINICAL MICROSCOPY BRANCH

Introduction to Blood Coagulation (1 hour)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist Course (Basic)

PLACE: Classroom

REFERENCES:

Beck, William S. Hematology. M.I.T. Press, 1974.
Bigge, Rosemary and Macfarlane, R.G. Human Blood Coagulation. 3d ed. Philadelphia: F.A. Davis Company, 1972.

STUDY ASSIGNMENT: After class, study M 08-280-045-1 and M 08-280-045-2.

STUDENT UNIFORM AND EQUIPMENT: None

TOOLS, EQUIPMENT, AND MATERIALS: During class issue M 08-280-045-1 and M 08-280-045-2 one per student as indicated in lesson plan.

PERSONNEL: One instructor (92B4H, 92B3H, or 92B2H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, and eraser

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Lecture

I. **INTRODUCTION** (5 min)

A. Opening statement: One of the major functions of blood is to maintain tissue stability, thereby keeping the internal environment of the body constant so that normal physiologic processes may occur. In order to maintain this stability, blood must remain a fluid. Should an abnormal vascular condition occur, certain changes take place which stop the flow of blood through a vessel wall. These physiologic changes are known as hemostasis.

B. Objectives

1. List the three systems which are involved in hemostasis.

2. List the three phases of coagulation in proper sequence.

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*This lesson plan supersedes LP 08-280-045, 075.

C. Class Procedure and Lesson Tie-in

1. First of five hours on hemostasis and blood coagulation.

2. Conferences, when necessary, to solve individual problems.

II. EXPLANATION (35 min)

A. Hemostasis

1. Definition: process by which the body stops spontaneous or induced bleeding or hemorrhage.

2. Hemostatic mechanism is comprised of three systems:

   a. Vascular system -- composed of arteries, veins, and microcirculation. All must be structurally and functionally normal.

   b. Platelet system -- to aid in hemostasis platelets must be present in adequate numbers and they must function properly.

   c. Coagulation system -- composed of at least twelve plasma coagulation factors. These factors make up two separate but interdependent systems:

      (1) Intrinsic system -- plasma thromboplastin is generated by the reaction of several plasma coagulation factors.

      (2) Extrinsic system -- this system is triggered by tissue injury which releases tissue thromboplastin.

B. Blood Coagulation Factors

1. All are proteins except calcium.

2. All, except thromboplastin, can be found in the plasma portion of the circulating blood.

3. Labeled by Roman numerals.

NOTE: Pass out M 08-280-045-1, one per student.

C. Hemostatic Mechanism -- Sequence of Reactions

1. Injury to vessel and release of tissue thromboplastin

2. Exposure of subendothelial tissue (collagen) to circulating platelets

3. Platelet adhesion to collagen with subsequent aggregation and release of serotonin, epinephrine, A.D.P. and platelet factor 3

4. Vessel constriction by serotonin

5. Activation of both intrinsic and extrinsic pathways leading to the formation of thrombin

6. Conversion of fibrinogen to fibrin by thrombin

7. Formation and stabilization of fibrin clot

D. Intrinsic Coagulation System

   1. Phase 1 -- formation of plasma thromboplastin

      a. Requirements

         (1) Foreign surface

         (2) Platelets -- platelet factor 3

         (3) Plasma coagulation factors

            (a) XII - Hageman factor

            (b) XI - PTA

            (c) IX - PTC

            (d) X - Stuart factor

            (e) VIII - Antihemophilic factor

            (f) V - Proaccelerin

         (4) Calcium

      b. Schematic diagram

XII
XI
X $\xrightarrow[\text{Calcium}]{\text{V}}$ Plasma thromboplastin
XI
VIII
P.F. 3

QUESTION: What is the end product of phase I in coagulation?

ANSWER: Plasma thromboplastin.

2. Phase 2 - formation of thrombin
    a. Requirements
        (1) Prothrombin
        (2) Thromboplastin: either plasma or tissue
        (3) Thrombin: from extrinsic pathway
        (4) Plasma coagulation factors
            (a) Factor V
            (b) Factor X
        (5) Calcium
    b. Schematic diagram

$$\text{Prothrombin} \xrightarrow[\text{Ca}^{++},\ \text{V},\ \text{X}]{\text{Plasma thromboplastin}} \text{Thrombin}$$

3. Phase 3 -- formation of fibrin
    a. Requirements
        (1) Fibrinogen
        (2) Thrombin
        (3) Factor XIII
    b. Schematic diagram

$$\text{Fibrinogen} \xrightarrow[\text{XIII}]{\text{Thrombin}} \text{Fibrin}_{\text{(stabilised)}}$$

NOTE: Issue M 08-280-045-2, one per student.

E. Extrinsic System

1. By-passes phase I

2. Thromboplastin derived from injured tissue

3. Requirements

   a. Factor VII

   b. Factor V

   c. Factor X

   d. Calcium

   e. Prothrombin

4. Schematic diagram

$$\text{Prothrombin} \xrightarrow[\text{Ca}^{++},\ \text{V, VII, X}]{\text{Tissue thromboplastin}} \text{Thrombin}$$

5. Phase 3: same as intrinsic systems

QUESTION: What coagulation factors are common to both systems?

ANSWER: Factors V and X.

F. Laboratory Testing

1. Vascular system

   a. Bleeding time - Ivy template

   b. Tourniquet test

2. Platelet system

   a. Platelet count

   b. Platelet aggregation

   c. Platelet factor 3 availability

3. Coagulation system
    a. Prothrombin time (P.T.)
    b. Partial thromboplastin time (P.T.T.)
    c. Fibrinogen level - thrombin time
    d. Specific factor tests

III. SUMMARY

A. Review of Main Points
    1. Hemostasis
    2. Three systems comprising the hemostatic mechanism
    3. Blood coagulation factors
    4. Sequence of reactions in hemostasis
    5. Intrinsic system
    6. Extrinsic system
    7. Laboratory testing

B. Closing Statement: A complete understanding of hemostasis will enable you to apply specific laboratory tests to the solution of coagulation problems and in so doing reduce the testing time and increase the accuracy of diagnosis.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY M 08-280-045-1
LABORATORY SCIENCES DIVISION 104
CLINICAL MICROSCOPY BRANCH

Blood Coagulation Factors

NUMBER	PROPER NAME	PHASE FOUND IN	ORIGIN
I	Fibrinogen	Phase IV	Liver to plasma
II	Prothrombin	Phase III	Liver to plasma
III	Thromboplastin	Phase II & III	Tissue & plasma
IV	Calcium (CA^{++})	All Phases	*
V	Proaccelerin	Phase II & III	Plasma Protein
VII	Proconvertin	Phase III	Plasma Protein
VIII	Antihemophilic factor (AHF)	Phase II	Plasma Protein
IX	Plasma Thromboplastin Component (PTC)	Phase II	Plasma Protein
X	Stuart factor	Phase II & III	Plasma Protein
XI	Plasma Thromboplastin Antecedent (PTA)	Phase I	Plasma Protein
XII	Hageman factor	Phase I	Plasma Protein
XIII	Fibrin Stabilizing factor	None	Plasma Protein

The term "Factor VI" has been deleted when it was found to be an activated form of factor V, Proaccelerin.

*Calcium is found throughout the body and can never become sufficiently deficient to affect the coagulation mechanism.

Blood Coagulation Factors in General

Factors, except Calcium and Thromboplastin, are proteins of the general group termed globulins. They can be found in the plasma portion of the circulating blood. Their production is under genetic control, hence deficiencies of these factors are often hereditary. Thromboplastin is a protein that must be liberated from tissue cells or generated in the plasma.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-017
LABORATORY SCIENCES DIVISION 075
CLINICAL MICROSCOPY BRANCH

Bleeding Time and Coagulation Time (1 hour)

COURSES PRESENTED TO: 311-92B20, Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES: TM 8-227-4

STUDY ASSIGNMENT: After class read and review TM 8-227-4, pp 6-6 to 6-8

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: None

PERSONNEL: One primary instructor (MOS 92B2H, 92B3H, or 92B4H)

INSTRUCTIONAL AIDS: Chalk, chalkboard and eraser.

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

I. INTRODUCTION (5 min)

A. Opening Statement: When a housewife cuts her finger on broken glass, her reaction typifies the blind faith the average person has in the body's ability to stop the flow of blood. For the housewife, the discomfort due to her cut finger is her major worry. The technician on duty in the hematology section of the laboratory performs daily bleeding and coagulation determinations. Reliable results may hasten the doctor's arrival at a diagnosis and establish the necessary treatment for the patient. The results of inaccurate reports are obviously potentially injurious to the patient. After this hour of instruction, placed in the same situation as the housewife, I am sure that you will be more interested in how much you bleed and how long the bleeding continues than how much it hurts!

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*This lesson plan supersedes LP 08-280-017, 014.

B. Objectives

Explain and discuss the principles and procedures used in determining the bleeding time and coagulation time; list the normal range and sources of error for each determination.

C. Class Procedure and Lesson Tie-in

1. This is the second hour of a four hour block of instruction on coagulation.

2. Will be followed by a practical exercise in accordance with individuals needs.

3. Conferences as necessary to solve individual problems.

II. EXPLANATION (35 min)

A. Bleeding Time (Ivy's Method)

1. Principle - the time necessary for a small standardized wound to stop bleeding.

2. Equipment

a. Surgical blade

b. Filter paper

c. Stop watch

d. Blood pressure cuff (sphygmomanometer)

3. Technique

a. Blood pressure cuff 40 mm pressure

b. Clean forearm just below elbow with 70% alcohol and allow to dry.

c. Make three incisions 3 mm deep and 3 mm long with surgical blade avoiding superficial veins. Use plastic template.

d. Start watch.

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    e. At 15 second intervals, blot with filter paper.

    f. When blood flow ceases - stop watch.

  4. Normal range (up to 7 minutes)

  5. Prolonged

    a. Thrombocytopenia

    b. Defect in vascular integrity

QUESTION: What is the standardized depth of the wound made while determining the bleeding time?

ANSWER: Three millimeters.

  6. Sources of error

B. Bleeding Time (Dukes Method)

  1. Principle - (same as Ivy's)

  2. Technique

    a. Same equipment (except for blood pressure cuff)

    b. Stick in ear lobe 15-20 mm above fatty portion with surgical blade. Only one incision is required.

    c. Time every 30 seconds.

  3. Normal range (1 to 3 minutes)

  4. Sources of error

C. Coagulation Time - (Lee-White Method)

  1. Principle - Time necessary for blood to clot in the absence of tissue factors under normal conditions

  2. Equipment

    a. Venipuncture equipment

    b. Normal saline

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    c. Three acid cleaned test tubes (13 x 100 mm)
    d. Incubator, 37° C
    e. Stop watch.

3. Technique
    a. Label tubes 1, 2, 3.
    b. Rinse all glassware in normal saline.
    c. Do atraumatic venipuncture, using two syringe method.
    d. Start watch.
    e. Put 1.0 ml of blood in tubes, reverse order.
    f. Incubate for 5 minutes.
    g. Tilt tubes every 30 seconds.
    h. When all tubes have clotted, stop watch.
4. Normal range
    a. 37° C - 5 to 15 minutes
    b. Room temperature - up to 20 minutes
5. Prolonged
    a. Hemophilia
    b. Factor IX
    c. PTA deficiency
    d. Hageman's trait

QUESTION: How much blood is placed into each of the tubes when performing a coagulation time determination?

ANSWER: One milliliter.

6. Sources of error

D. Questions From Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Bleeding Time

2. Coagulation Time

B. Closing Statement: To be of value to the requesting physician the bleeding and coagulation time determinations are listed among those laboratory procedures that require faultless technique on the part of the laboratory technician. It will take hours of practice for you to develop the ability to perform these procedures correctly and report the results with confidence to the physician.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *IM 08-280-017
LABORATORY SCIENCES DIVISION 075
CLINICAL MICROSCOPY BRANCH

Bleeding Time and Coagulation Time

When the housewife cuts her finger on broken glass, her reaction typifies the blind faith of the average person in the body's ability to stop the flow of blood. For the housewife, the discomfort of the cut is her major worry. The technician on duty in the hematology section of the laboratory will perform daily bleeding and coagulation time determinations. Reliable results may hasten the doctor's arrival at a diagnosis and establish the necessary treatment for the patient. The results of inaccurate reports are obviously potentially injurious to the patient. After this hour of instruction, placed in the same situation as the housewife, I am sure that you will be more interested in how much you bleed and how long the bleeding continues than how much it hurts!

When, because of a history or clinical symptons the medical officer suspects that the patient has a coagulation defect, he will often order a battery of basic coagulation studies to be done. The first of these - the bleeding time determination - is defined as the length of time required for small, standardized wounds (introduced into a capillary bed) to stop bleeding. The results of this procedure depend upon the elasticity of the skin, and capillaries, the efficiency of the tissue fluids, and the chemical and mechanical action, as well as the number and quality of the thrombocytes or platelets.

Equipment necessary for the performance of the bleeding time determination by Ivy's method includes a soft grade of filter paper, blood pressure cuff, stopwatch, surgical blade, 70% alcohol, and gauze.

The blood pressure cuff is put on the arm above the elbow and is pumped up to 40 mm pressure. Then a small puncture is made with the lancet, being sure not to puncture a vein in this process. The stopwatch is started immediately after the puncture is made. A puncture of proper depth will result in a free flow of blood, without the necessity of squeezing the bleeding site. At intervals of 15 seconds the drops of blood from the wound are gently absorbed with the filter paper; care must be taken not to touch the skin. When blood has ceased to appear at the puncture site, the watch is stopped. The time elapsed between the puncture and the cessation of bleeding is the bleeding time and is reported in 15 second increments.

The normal range established for the bleeding time performed by Ivy's method is up to 7 minutes. For this test to have any significance to the medical officer, it is essential that standardized puntures be made and the procedure followed exactly.

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*This instructor's manuscript supersedes IM 08-280-017, 014.

A bleeding time shorter than 1 minute usually indicates improper performance of the puncture. A prolonged bleeding time, on the other hand, may indicate improper performance, but it may also be an indication of such conditions as thrombocytopenia or a defect in the patients' vascular integrity. Therefore, when abnormal results are obtained, additional determinations should be done (because of the difficulty of producing a standardized wound) to make sure the abnormal results are valid.

The standardized depth of the wounds made while determining the bleeding time is three millimeters. This point should be emphasized, since all conditions of the determination must be standardized as closely as possible for results to be trustworthy. Too deep a puncture may go through a capillary bed and enter a larger vessel, giving an extra flow of blood and an apparently prolonged bleeding time. A wound too shallow, which barely goes through the full thickness of the skin, gives a scanty blood flow or even no bleeding at all. Another source of error is the destruction of the partially formed clot by wiping the blood off with filter paper. Error may also be introduced by squeezing the tissue at the puncture site. Other error factors include the temperature and thickness of the skin at the site selected for puncture.

Another method for determining bleeding time is Dukes' procedure. The principle is the same as Ivy's; however, instead of using the forearm for the puncture site, the ear lobe or finger is used. A blood pressure kit is not needed, but a glass slide or small test tube is required on this method if done on the ear lobe.

A small puncture wound 3 millimeters in depth is made over the lateral aspect of the fingertip or the rim of the lower part of the ear lobe. (The test tube or microscope slide is used to stabilize ear lobe in this method.) Timing is the same as in Ivy's method and the results are reported the same way.

The normal range established for the bleeding time by Dukes' method is 1 to 3 minutes. Again a standardized puncture is required. Error sources are essentially those encountered when using the Ivy method of determining bleeding time.

Another basic procedure is the determination of the coagulation time. This is considered to be the interval of time required for blood to clot in the absence of tissue factors and under controlled conditions. Although there are many procedures, we will discuss only one. The Lee-White coagulation time. This procedure involves the use of venous blood in its performance. Since tissue thromboplastin can affect the results drastically, the venipuncture by which a specimen is obtained must be as "clean" and as atrumatic as possible.

Equipment needed to perform the Lee-White coagulation time determination includes a stopwatch, 2 needles, 2 syringes, gauze, 70 percent alcohol, three acid cleaned test tubes, 100 mm in length and 11 mm internal diameter, and a 37°C incubator.

Though the technique is simple, the technician must be meticulous in following instructions exactly. Conditions under which the determination is performed must be standardized, and deviation from the standard conditions at any stage of the procedure will introduce error, making it difficult to evaluate the accuracy of the results. Label three clean tubes of the proper size 1, 2, and 3. Rinse these tubes and the syringes to be used (if glass) in normal (0.85 percent) saline. The vein is selected and the venipuncture site prepared before the puncture is made; the tourniquet is applied just prior to venipuncture. The needle must enter the vein quickly and cleanly to prevent, as much as possible, tissue thromboplastin from entering the syringe with the blood. The two-syringe technique should be utilized. This is done first by drawing two milliliters of blood, removing the syringe and discarding it. Attach a second syringe and withdraw four milliliters of blood. Start the watch as soon as blood appears in the hub of the second syringe. The tourniquet is released and the needle withdrawn from the vein. The needle is removed from the syringe and 1 ml of blood is placed into each tube in reverse order. The last milliliter is discarded since it probably will contain some tissue thromboplastin in spite of the care taken during venipuncture. By filling the tubes in this way, the blood appearing last in the syringe is checked for coagulation last. The tubes are then placed in a 37°C incubator. They should not be agitated as this will cause hastened coagulation. They are left in the incubator for five minutes. At the end of that time, tube #1 is tilted at 30-second intervals until it can be inverted without loss of the blood it contains. When this can be done, tube #2 is treated the same, then tube #3. When tube #3 coagulates, the watch is stopped. The time shown on the watch is the coagulation time and should be reported in minutes and half-minutes. When the 37°C incubator is utilized the normal range is established as 5 to 15 minutes. Occasionally this procedure must be performed at the patient's bedside where no incubator is available. Under these room temperature conditions, the normal range is said to be up to 20 minutes.

A prolonged coagulation time indicates that the patient has some type of bleeding tendency, with the severity of the condition at least roughly correlated with the amount of prolongation in excess of the normal range. Hemophilia is probably the best known condition giving abnormally long coagulation times. However, there are other defects in the coagulation mechanism which also lengthen the time required for the blood to clot. Among these are deficiencies in Factor IX or PTA; a patient with Hageman's disease, either the trait or the condition, will also have a prolonged coagulation time. More commonly encountered in the laboratory is the patient on heparin anticoagulant therapy, who shows an abnormally long clotting time. The amount of prolongation enables the medical officer to evaluate the heparin treatment.

The amount of blood put into each of the tubes while performing a coagulation time is one milliliter. This point is stressed to help you realize the importance of standardizing conditions and controlling, so far as possible, the sources of error. Some of the common causes of error include a failure to rinse the tubes and syringe (if glass) in normal saline just before use, and using tubes which are too small or too large in diameter. A traumatic venipuncture, which introduces tissue thromboplastin into the blood, will shorten the coagulation time by an unknown amount. Agitation of the specimen and excess exposure to the air during any part of the procedure also shortens the time of coagulation, and could conceivably give the medical officer a false sense of security in the case of a patient who actually had a coagulation defect. Other sources of error, against which precautions must be taken, are the presence of bubbles (in the syringe or the test tube), using dirty glassware, leaving the tourniquet in place too long, and incubation at an improper temperature. These factors can cumulatively account for very large variations and inaccuracies in the final results.

During this hour we have discussed the principle, necessary equipment, procedural technique, normal values, and sources of error of two of the basic coagulation mechanism studies: bleeding time and coagulation time. We have also correlated abnormal results in each of these procedures with specific coagulation defects.

To be of any value to the doctor requesting them, the bleeding and coagulation time determinations are listed among those laboratory procedures that require faultless technique on the part of the laboratory technician. It will take hours of practice for you to develop the ability to perform these procedures correctly and report the results with confidence to the physician.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-018
LABORATORY SCIENCES DIVISION 078
CLINICAL MICROSCOPY BRANCH

Thrombocyte Count and Prothrombin Time (1 hour)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic)

PLACE: Classroom

REFERENCES:

TM 8-227-4
Beck, William S. Hematology. M.I.T. Press, 1974.
Biggs, Rosemary and Macfarlane, R. G. Human Blood Coagulation. 3d ed. Philadelphia: F. A. Davis Company, 1972.
Davidsohn, I., and Henry, J. B. Todd-Sanford Clinical Diagnosis by Laboratory Methods. 14th ed. Philadelphia: W. B. Saunders Company, 1969.
Miale, John B. Laboratory Medicine Hematology. 3rd ed. St. Louis: C. U. Mosby Company, 1967.

STUDY ASSIGNMENT: Prior to class study TM 8-227-4, chap 6, pp 6-10, 6-11, 6-17, and 6-18.

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT AND MATERIALS: None

PERSONNEL: One primary instructor (MOS 92B3H or 92B4H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, eraser, overhead projector, transparancies, and VT-318.

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

I. INTRODUCTION (5 min)

A. Opening Statement: Blood coagulation is a complex phenomenon involving twelve factors interacting in three "phases" of action. An absence of, or deficiency in any one or more of these factors may result in a defective coagulation mechanism. There are many tests which aid in determining the identity of a defect and locating its position in the coagulation system. Two of the tests employed for this purpose are the thrombocyte (platelet) count and the prothrombin time.

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*This lesson plan supersedes LP 08-280-018, 075.

B. Objectives

1. State the origin and list the functions of platelets.

2. List the functions of prothrombin and the purpose of performing the prothrombin time.

3. List the steps in the procedures and the sources of error in performing the direct platelet count, platelet estimate, and prothrombin time.

4. List the correct units of report and normal values.

C. Class Procedure and Lesson Tie-in

1. This is the third hour in a three hour block of instruction.

2. Conferences when necessary to solve individual problems.

II. EXPLANATION (30 min)

A. Thrombocytes (Platelets)

1. Description

a. Origin: Megakaryocyte

(1) Large nucleated cell in bone marrow

(2) Diameter -- 50-100 microns (u)

(3) Not normally seen in peripheral blood

(4) Fragmentation of cytoplasm

(5) Platelet life span - 8 to 11 days

b. Size and shape

(1) Round, oval, or rod shaped

(2) Normal -- 1-3 microns

(3) Giant platelets

(a) 8 - 10 microns

(b) May be confused with lymphocytes

c. Staining characteristics with Wright's Stain

(1) Lilac to light blue cytoplasm

(2) Centrally located red-purple azurophilic granules

(3) Anucleated

2. Functions

a. Mechanical plug of wound

(1) Platelet adhesiveness

(2) Aggregation

(3) "Viscous metamorphosis"

b. Clot retraction

(1) Measures thrombocyte activity

(2) Adequate if greater than 100,000 intact platelets/mm^3

(3) Deficient if less than 100,000 intact platelets/mm^3

c. Chemical action

(1) Serotonin - vasoconstrictor

(2) Platelet factor 3 - necessary for thromboplastinogenesis

3. Enumeration and estimation

a. Rees-Ecker method - direct count

(1) Principle

(2) Equipment

(a) Venipuncture equipment

NOTE: Capillary blood can be used, but anticoagulated (EDTA) venous blood is preferred.

- (b) RBC pipet and aspirator
- (c) Hemacytometer
- (d) Petri dish and damp gauze
- (e) Microscope

(3) Reagent: Rees-Ecker diluent

- (a) Anticoagulant - sodium citrate
- (b) Preservative - formalin
- (c) Stain - Brilliant Cresyl Blue

(4) Procedure

- (a) Rinse pipet with diluent
- (b) Draw blood to 0.5 mark of RBC pipet
- (c) Dilute with diluent to 101 mark

QUESTION: What is the dilution factor for the Rees-Ecker platelet test?

ANSWER: The dilution factor is 200.

- (d) Shake for 5 minutes
- (e) Discard first 4 drops
- (f) Load both sides of the hemacytometer
- (g) Place in Petri dish
  - 1 Damp gauze - prevents evaporation
  - 2 Cover with dish top
- (h) Let settle 15 minutes
- (i) Count platelets (blue refractile bodies)
  - 1 High power objective
  - 2 Reduced light
  - 3 Center 1 sq mm on both sides of hemacytometer

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NOTE: Demonstrate proper square; this is on chalkboard.

QUESTION: What is the area factor for the Rees-Ecker platelet determination?

ANSWER: The area factor is 1/2.

(5) Calculation:

platelets/mm^3 = number of platelets counted X Depth Factor X Area Factor X Dilution Factor

(6) Sources of error

(a) Artifacts confused with platelets

1 Dirty glassware - clean carefully

2 Dirty diluent - filter before use

(b) Improper dilution

(7) Unit of report -- platelets per cu mm

(8) Normal values -- 150,000 - 350,000 platelets/mm^3

b. Platelet estimate - indirect

(1) Principle

(2) Equipment - same as leukocyte differential

(3) Reagents - same as leukocyte differential

(4) Procedure

(a) Perform normal differential

(b) Observe number of platelets

1 Under oil immersion

2 At least 5 fields of approximately 200 RBC's

(5) Reporting platelet estimate

(a) Decreased - less than 10 platelets/oil immersion field

(b) Normal - 10 to 12 platelets/oil immersion field

(c) Increased - greater than 12 platelets/oil immersion field

(6) Source of error

(a) Improper area

(b) Precipitated stain

B. Prothrombin Time (Quick's One Stage Modification)

1. Coagulation review of extrinsic system

QUESTION: What is the source of thromboplastin in the extrinsic system?

ANSWER: Injured tissue.

QUESTION: What is the product of phase II and from what is it formed?

ANSWER: The product of phase II is thrombin which is formed from prothrombin.

QUESTION: What is the product of phase III and from what is it formed?

ANSWER: The product of phase III is fibrin which is formed from fibrinogen.

2. Importance

a. Measures extrinsic system

b. Measures activity of phase II

c. Monitors coumarin anticoagulant therapy

d. Diagnosis and prognosis of liver disease and vitamin K deficiency.

3. Principle

4. Technique

a. Manual

(1) Equipment

(a) Venipuncture equipment

(b) 0.1 & 0.2 ml serological pipets

(c) Kahn tubes (12 X 75 m)

NOTE: All glassware must be acid cleaned and used only for prothrombin times.

(d) Stopwatch

(e) Wire loop, #22 nichrome

(f) Dry block, 37°C

(2) Reagents

(a) Anticoagulant - 0.1M sodium citrate

(b) Simplastin

1 Tissue thromboplastin

2 Calcium

(c) Diagnostic control plasma -- normal human plasma

(3) Procedure

(a) Collect blood with the least possible tissue damage

(b) Mix exactly 4.5 ml of blood with 0.5 ml 0.1M sodium citrate (9:1 ratio)

NOTE: Blood must be tested within two hours after collection or it will lose activity. If not tested immediately, refrigerate at 4°C.

(c) Centrifuge at 1700 rpm for ten minutes

(d) Separate plasma from cells and place plasma at 37°C for five to ten minutes

(e) Reconstitute reagents

(f) Place 0.2 ml simplastin in Kahn tube (incubate at 37°C - three minutes)

(g) Add (blow in) 0.1 ml patient's plasma simultaneously starting stopwatch

(h) Read reaction

1 Tilt tube - 1 to 2 times per second

2 Wire loop - 1 to 2 times per second

(i) Record time clot forms to nearest 5/10 second

(j) Control-run prior to patient testing

(k) Repeat both control and patient

b. Automated

(1) Equipment

(a) Venipuncture equipment

(b) Fibrometer with cups and pipets

(2) Reagents - same as manual

(3) Procedure - refer students to video tape

5. Unit of report

a. Seconds and 5/10 of a second

b. Percent activity - from manufacturer's activity curve

6. Normal values - 12 to 15 seconds

7. Sources of error

a. Improper timing

b. Improper temperature

c. Dirty glassware

d. Delay over two hours in running tests

e. Bad reagents

f. Pipeting error

C. Questions From Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Thrombocytes

2. Prothrombin Time

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B. Closing statement: The thrombocyte count and prothrombin time determination require a high degree of accuracy. A request for these determinations indicates suspicion of a blood coagulation problem. Only by meticulous attention to detail can the technician obtain the necessary accuracy when performing these tests.

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LABORATORY SCIENCES DIVISION 078
CLINICAL MICROSCOPY BRANCH

Thrombocyte Count and Prothrombin Time (1 hour)

Blood coagulation is a complex phenomenon involving 12 factors interacting in three "phases" of action. An absence of, or deficiency, in any of these factors, may result in a defective coagulation mechanism. There are many tests which aid in determining the identity of a defect and locating its position in the coagulation system. Two of the tests employed for this purpose are the thrombocyte (platelet) count and the prothrombin time.

J. H. Wright, in 1906, concluded that platelets are detached portions of the cytoplasm of megakaryocytes. These are extremely large cells, 50-100 microns in diameter, found in the bone marrow, lungs, and spleen. Because of their large size they are not normally found in peripheral blood. In the bone marrow the megakaryocytes are so situated to the blood vessels that small fragments of their cytoplasm are pinched off and carried into the blood stream. By this process all of the cytoplasm is broken away until only the nucleus remains to degenerate. The life span of the platelets, themselves, has been estimated to be from 8 to 11 days.

Platelets are round, oval, or rod shaped and normally 1 to 3 microns in diameter, in anticoagulated peripheral blood smears. In capillary smears the platelets may become elongated or have sharp projections. Giant platelets, from 8 to 10 microns, may be seen when blood is being actively regenerated. The inexperienced technician must inspect these carefully so as not to confuse them with lymphocytes.

After staining with Wright's stain, the platelets have a lilac to light blue cytoplasm and red-purple azurophilic granules. The azurophilic granules are sometimes centrally located giving a nuclear appearance; however, the granules do not take nuclear stains. Also a nucleus has never been demonstrated in platelets.

Though small and fragile, platelets have several very important functions. One property of platelets is their adhesiveness. When a blood vessel is injured, the platelets come in contact with a foreign environment. This contact causes the platelets to become "sticky" and adhere to the blood vessel and surrounding tissues. The platelets also adhere to one another and aggregate at the injury site to form a platelet plug. "Viscous metamorphosis" then takes place with the platelets losing their shape and forming an amorphous hyaline-like clump. Although the platelets undergo this change, the platelets membrane and cell integrity remains intact.

When whole blood is allowed to clot, it can be observed that the clot decreases in size and clear serum is expressed. This phenomenon is known as clot retraction and is dependent upon the number of intact platelets

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*This instructor's manuscript supersedes IM 08-280-018, 075.

present. Within limits, the higher the platelet count, the more complete the clot retraction. Therefore, if the platelet count falls below 100,000/mm^3 clot retraction is poor. Clot retraction is essential in the formation of a hemostatic plug and is a good measure of thrombocyte activity.

In addition to mechanical plugging of wounds and their role in clot retraction, platelets exert a chemical action in hemostasis. Platelets release a powerful chemical, serotonin, which acts as a vasoconstrictor at the injury site. Platelets also contain platelet factor 3, a chemical factor necessary for thromboplastinogensis.

The unique characteristics of platelets make them difficult to count. Platelets may be enumerated by either a direct or indirect method. In the direct method they are counted on a hemacytometer as in the white or red blood cell counts. The indirect method involves a determination of the number of platelets in proportion to the number of red blood cells.

The Rees-Ecker method is a direct procedure. In this method, venous or capillary blood (venous blood gives more reproducible results) mixed with an anticoagulant is mixed in a red blood cell pipet with a diluent, and the platelets counted in a hemacytometer. The Rees-Ecker diluent contains an anticoagulant (sodium citrate), a preservative (formalin), and a stain (brilliant cresyl blue).

In the Rees-Ecker method the red cell pipet is rinsed by filling the pipet with diluent to the 101 mark and then expelling the diluent. By rinsing the pipet, its inner surface is coated, thereby preventing the platelets from adhering to its sides. Anticoagulated blood is then drawn to the 0.5 mark and diluted with Rees-Ecker diluent to the 101 mark, resulting in a final dilution of 1:200. The pipet is thoroughly mixed for 5 minutes and the first four drops from the pipet are discarded. Both sides of the hemacytometer are loaded and it is then placed in a covered Petri dish where the platelets are allowed to settle for 15 minutes. To prevent evaporation, a damp gauze pad should be placed in the Petri dish along with the hemacytometer.

The platelet count is performed using the high power objective and reduced light. The platelets appear as blueish, highly refractive, round, oval, or elongated particles when viewed through the microscope. The number of platelets in the center square millimeter, of both chambers, of the hemacytometer, are counted. Since the two center sq mm's are counted, the area factor for the platelet determination becomes 1/2.

The calculation of the number of platelets per cu mm utilizes the same basic formula as employed in both the WBC and RBC counts: platelets per cu mm equal the number of platelets counted times the depth factor (10) times, the area factor (1/2) times, and the dilution factor (200) times.

The direct platelet determination has several possible sources of error. The most obvious of these is improper dilution of the specimen. Another source of error is confusing artifacts with platelets. Dirty glassware and contaminated diluent are the main sources of these artifacts. Careful cleaning of the glassware and filtering of the diluent prior to use will help avoid this source.

Various normal ranges have been established depending upon which type of determination is being used. For the Rees-Ecker procedure the normal range for the platelet count has been established as 150,000 to 350,000 platelets per cu mm.

It is possible to recognize a significant increase or decrease of platelets while performing a differential while cell count on a peripheral blood smear. This is the indirect method. A venous blood specimen is preferred to a capillary specimen because of possible dilution with tissue fluid and direct contact with the tissue surface.

Upon completion of a routine white cell differential count, the technician may perform the platelet estimate. The number of platelets in at least five oil immersion fields of approximately 200 red cells are observed in order to obtain a rough estimate as to their number. A normal estimate has an average of 10 to 12 platelets per oil immersion field. More than 10 to 12 platelets per oil immersion field is indicative of an increase and less than 10 to 12 platelets per oil immersion field a decrease. If there is a significant decrease in platelets it is wise to perform a platelet count.

When performing a platelet estimate, the technician should be careful to avoid two sources of error. The first of these is in not performing the estimate in fields of approximately 200 red blood cells; the other is not to confuse precipitated stain with platelets.

In the introductory lecture on coagulation we saw how the end product, fibrin, is derived by proceeding through three phases. In the extrinsic coagulation system, phase I is by-passed. In phase II, prothrombin acts with calcium, factors V, VII, X and the thromboplastin from injured tissue to form thrombin. In the final phase, fibrinogen acts with thrombin from phase II to form the final coagulation end product, fibrin.

The prothrombin time is important as a measure of the extrnisic system. It not only measures prothrombin activity, but also the activity of factors V, VII and X in phase II of coagulation. The prothrombin time is also useful in monitoring anticoagulant therapy, the diagnosis and prognosis of liver disease, and in vitamin K deficiency.

The principle of the prothrombin time may be stated as: an extrinsic source of thromboplastin and calcium is added to the patient's plasma and the one stage prothrombin time is performed. The test measures those coagulation factors involved in the extrinsic clotting system, namely factors I, II, VII, X and V.

There are two methods which may be employed to perform a prothrombin time, the manual and the automated. The manual method will be discussed first and requires the assemblage of certain equipment and reagents.

The equipment necessary for the manual technique is: normal venipuncture equipment, 0.1 and 0.2 ml serological pipets, 12 X 75 mm Kahn tubes, a stopwatch, a #22 nichrome wire loop, and a 37°C dry heating block. The glassware used in this procedure should be acid cleaned and used only for performing prothrombin times. In addition to the equipment, there are specific reagents utilized.

The first reagent is a calcium binding anticoagulant, 0.1$\underline{M}$ sodium citrate. The other two reagents are Simplastin, which supplies tissue thromboplastin and calcium; and Diagnostic Control Plasma, which serves as a normal control. The latter two reagents must be reconstituted according to manufacturer's instructions.

Prior to beginning the procedure, a blood specimen is collected as nontraumatically as possible to prevent contamination by tissue fluids. Exactly 4.5 ml of the blood specimen is thoroughly mixed with 0.5 ml of the 0.1$\underline{M}$ sodium citrate. The specimen is then centrifuged at 1700 rpm for 10 minutes and the plasma separated from the cells and placed at 37°C for 5 to 10 minutes (if the plasma is not to be used immediately refrigerate at 4°C).

The commercial reagents are reconstituted with sterile water following manufacturers instructions. Next 0.2 ml of Simplastin is pipetted into a Kahn tube and incubated at 37°C for 3 minutes. After three minutes, 0.1 ml of patient's plasma is added to the 0.2 ml of Simplastin. The stopwatch is simultaneously started as this step is being accomplished. Immediately, mix the constituents and time the reaction either by tilting the tube 1 to 2 times per second, or by drawing a #22 nichrome loop through the tube's contents 1 to 2 times per second until a clot forms. Upon the appearance of the clot, stop the watch and record the time to the nearest 5/10 second. Controls, using the Diagnostic Control Plasma, should be run before patient tests to insure that all reagents are properly functioning. In order to insure validity, both the patient and control procedures should be run in duplicate with duplicate values agreeing within 0.3 second.

Due to the importance of accuracy and reproducibility, several commercial companies have developed automated equipment for performing the prothrombin time. Though design of automated equipment may vary according to the manufacturer, the basic principle of each are the same.

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A fibrometer is employed to perform the prothrombin time. A built-in heating block maintains the temperature at a constant 37°C. An automatic pipetter dispenses the Simplastin and patient's plasma and simultaneously starts a timer. The end is electronically determined with the elapsed time for clot formation indicated on a readout. The prothrombin time is reported as seconds and 5/10 of seconds. The prothrombin time may also be expressed as per cent activity which may be calculated by applying the patient's prothrombin time (in seconds) to a prothrombin activity curve. This activity curve is supplied by the reagent manufacturer. Normal values for the prothrombin time is from 12 to 15 seconds. The automated system also has the advantage of utilizing plastic disposable cups and pipets. Further information on the automated procedure is available through numerous texts.

When performing the prothrombin time there are several sources of error the technician should be aware of. Some of the more important ones are: improperly timing the reaction; temperature variation from 37°C when incubating; dirty or scratched glassware which may accelerate clotting; a delay of over two hours before performing the test; pipetting errors; and bad reagents.

The thrombocyte count and prothrombin time determinations require a high degree of accuracy. A request for these determinations indicate suspicion of a blood coagulation problem. Only by meticulous attention to detail can the technician obtain the necessary accuracy when performing these tests.

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*LP 08-280-025
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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
CLINICAL MICROSCOPY BRANCH

Erythrocyte Morphology (2 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES:

TM 8-227-4

STUDY ASSIGNMENT: After class, read TM 8-227-4, chap 4, pp 4-1 thru 4-5.

STUDENT UNIFORM AND EQUIPMENT: Not Applicable

TOOLS, EQUIPMENT, AND MATERIALS: None

PERSONNEL: One instructor (MOS 92B2H), 92B3H, or 92B4H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, eraser, 35 mm transparencies (see Annex A)

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

FIRST HOUR

I. INTRODUCTION (5 min)

A. Opening Statement: The leukocyte differential count is one of the most frequently performed determinations in the laboratory. Inherent in this procedure is the evaluation of erythrocyte morphology. Since abnormal erythrocyte morphology may indicate a serious illness, it is imperative that it be correctly evaluated and reported by the laboratory technician.

B. Objectives

1. Identify the stages of maturation of the erythrocyte.

2. Be able to recognize the characteristics and descriptive terminology used to identify and classify each of the normal and abnormal forms of erythrocytes.

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*This lesson plan supersedes LP 08-280-025, 075

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C. Class Procedure and Lesson Tie-in

   1. First two hours of a block of instruction on the evaluation of erythrocyte morphology.

   2. Conferences when necessary to solve individual problems.

II. EXPLANATION (80 min)

A. Origin of Erythrocytes

   1. At birth - Active red marrow in all marrow cavities.

   2. In adults - Localized in red marrow

B. Normal Red Cell Maturation

NOTE: As each of the different cell types are discussed, display the appropriate 35 mm transparency.

   1. Rubriblast - Pronormoblast

      a. Size - 14 to 19 microns

      b. Nucleus -

         1. Shape - Round or very slightly oval

         2. Morphology - Fine reticular chromatin, 1-2 faint nucleoli

      c. Cytoplasm

         1. Amount - scanty

         2. Color - deeply basophilic

   2. Prorubricyte - Basophilic normoblast

      a. Size - 10 to 15 microns

      b. Nucleus -

         1. Shape - Round

         2. Morphology - Coarse, dark chromatin; no nucleoli; sparse, distinct parachromatin.

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- c. Cytoplasm
    - 1. Amount - Sparse, but more abundant than in rubriblast
    - 2. Color - Moderately to intensely bosaphilic

3. Rubricyte - Polychromatic normoblast
    - a. Size - 8 to 10 microns
    - b. Nucleus
        - 1. Shape - Round
        - 2. Morphology - Coarse, dark chromatin; distinct parachromatin.
    - c. Cytoplasm
        - 1. Amount - Abundant in comparison to earlier stages.
        - 2. Color - Blue with orange tinges due to hemoglobin.
4. Metarubricyte - Orthochromic normoblast
    - a. Size - 7 to 10 microns
    - b. Nucleus
        - 1. Shape - Often round, sometimes irregular
        - 2. Morphology - Uniformly dark, dense chromatin.
    - c. Cytoplasm
        - 1. Amount - Abundant
        - 2. Color - Bluish buff to buff pink.

NOTE: The nucleated cells mentioned above are often referred to collectively as "NRBC's". They do not occur normally in the peripheral blood (except at birth)

5. Diffuse basophilic erythrocyte - Reticulocyte
    - a. First non-nucleated stage
    - b. Size - 7 to 9 microns

c. Color - Bluish buff to buff pink

d. Small number normally present in peripheral blood.

6. Mature Erythrocyte

QUESTION: What is the diameter of a normal mature erythrocyte?

ANSWER: Seven to eight microns.

a. Shape - Biconcave disc

b. Color - Buff pink with slight central pallor.

C. Abnormal Erythrocyte Morphology

1. Anisocytosis - Variation in size

a. Macrocytes - diameter 9 microns or more.

b. Microcytes - diameter 6 microns or less

QUESTIONS: Macrocytes and microcytes are seen in certain anemias. What do we mean by the term anemia?

ANSWER: We define anemia as a deficiency in the quantity and/or quality of red blood cells.

2. Poikilocytosis - Variation in Shape.

a. Sickle - like cells

b. Oualocytes - Elliptocytes

c. Spherocytes - "Microspherocytes"

d. Crenated cells - Treated as artifacts; not reported.

e. Irregularly contracted cells; bizarre forms.

NOTE: The category of "Irregularly contracted cells" (Bizarre forms") is used to collectively refer to any variations of cell shape not otherwise mentioned specifically.

SECOND HOUR

3. Hemoglobin variations

a. Hypochromic cells

- b. Target cells
- 4. Erythrocytic inclusions
  - a. Poly chromatic cells - Diffuse basophilia
  - b. Basophilic strippling
  - c. Howell - Jolly bodies - Dark violet; 1 micron or less in diameter.

QUESTION: Of the abnormalities discussed, which one is regarded as an artifact and not reported?

ANSWER: Crenated cells

- D. Evaluation of Morphology
  - 1. Method of Examination
    - a. Use oil immersion objective
    - b. Perform an evaluation during differential count
    - c. Scan slide further after differential
  - 2. Report Findings
    - a. Normal Findings (N/N)
      - 1. Normocytic - Normal cell size and shape.
      - 2. Normochromic - Normal hemoglobin content and coloration
    - b. Abnormal Findings
      - 1. Use specific terminology
      - 2. Enumerate NRBC's per 100 WBC's
      - 3. Grade degree of all other abnormalities

NOTE: Minor, but normal, variations of size and shape occur even on normal smears, as do target cells, although rather infrequently.

- a. Slight - Abnormal forms seen intermittenly
- b. Moderate - Few abnormal forms seen in most fields
- c. Marked - Several abnormal forms seen in almost all fields

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NOTE: Howell - Jolly bodies may be reported as "Present" or "Seen". Always consult local laboratory SOP for possible exceptions to these rules. Some laboratories may grade abnormalities as being from 1+ to 4+.

E. Questions from Students

III. SUMMARY (5 min)

A. Review of main points

1. Origin of erythrocytes

2. Normal red cell maturation

3. Abnormal erythrocyte morphology

4. Evaluation of morphology

B. Closing Statement: The evaluation of erythrocyte morphology is an integral part of every WBC differential. However, the ability to evaluate morphology correctly is the result of a great deal of study and serious concentration at the microscope. Do not take this task lightly, since your power of observation relative to erythrocyte morphology may be of invaluable assistance to the physician and his patient.

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ANNEX A

Instructional Aids

35mm Transparencies

Transparency Number	Description:
1.	Rubriblast
2.	Prorubricyte
3.	Rubricyte
4.	Metarubricyte
5.	Diffuse basophilic erythrocyte
6.	Erythrocyte, biconcave shape
7.	Erythrocyte, central pallor
8.	Macrocyte
9.	Microcyte
10, 11, 12	Sickle-like cells
13.	Ovalocyte
14.	Spherocyte
15.	Crenated eryythrocyte
16, 17, 18	"Bizarre forms"
19	Hypochromic erythrocyte
20	Target cell
21	Poly chromatic cell
22	Erythrocyte-basophilic strippling
23, 24	Erythrocyte- Howell-Jolly Bodies

NOTE: These transparencies are used in parts IIB and IIC.

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CLINICAL MICROSCOPY BRANCH

Erythrocyte Morphology

The leukocyte differential count is one of the most frequently performed determinations in the laboratory. Inherent in this procedure is the evaluation of erythrocyte morphology. Since abnormal erythrocyte morphology may indicate a serious illness, it is imperative that it be correctly evaluated and reported by the laboratory technician.

Before elaborating upon the morphology of erythrocytes, let us first consider their origin. At birth extramedullary erythropoiesis in the liver and spleen is negligible, but the red bone marrow is very active in erythropoiesis and fills all marrow cavities of the body. The newborn infant has no marrow reserve and if there is a need for increased cell production it must take place at extramedullary sites. As the body grows, the marrow cavities enlarge and less of the potential hemopoietic tissue is required. During this period, the red marrow is being gradually replaced by yellow or fatty marrow.

In the adult the yellow marrow has increased in quantity to the point that red marrow is found only in the proximal epiphyses of the long bones (femur, humerus), the ribs, skull bones, sternum, clavicles, scapulae, vertebrae, and flat pelvic bones. The yellow marrow acts as a hemopoietic reserve, since it is capable of rapid transformation to active red marrow should the need arise.

The kinetics of hemopoietic proliferation are complicated and not yet fully understood. Likewise, the exact mechanism by which red blood cells enter the peripheral circulation is not known. The maturation and morphology of erythrocytes from the most immature (blast) stage to the mature stage can be deliniated, however, the nomenclature recommended by the American Society of Clinical Pathologists will be used, and commonly used synonyms will be mentioned in addition to recommended terms.

The most immature form of the erythrocyte is the rubriblast (pronormoblast). This cell is about 14 to 19 microns in diameter. The nucleus is large and either round or very slightly oval with one or two faint nucleoli present. The nuclear chromatin is finely reticular, having a loose or spongy appearance.

The cytoplasm is scanty and stains a dark blue (deeply basophilic) with Wrights stain. Specific cytoplasmic granules are not found in erythrocytes, including rubriblasts.

The next stage in the erythrocytes development is known as the prorubricyte (basophilic normoblast). This cell is 10 to 15 microns in diameter with a round nucleus exhibiting coarse, dark chromatin with no nucleoli. More cytoplasm in relation to nuclear size, is present than in the previous stage.

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*This instructor's manuscript supersedes IM 08-280-025, 075

The cytoplasm is royal blue and only slightly less basophilic than the cytoplasm of the rubriblast.

The prorubricyte matures to become a rubricyte (poly chromatic normoblast), 8 to 12 microns in diameter. The nucleus is round and exhibits chromatin that is somewhat coarser and denser than that of the prorubricyte. Parachromatin, sparse in the prorubricyte, is distinct in the rubricyte. Cytoplasm, more abundant than in the prorubricyte, which is blue with an orange tinge, due to the first visible appearance of hemoglobin.

The metarubricyte (orthochromic normoblast) is the terminal nucleated stage in the erythrocyte's development. This cell is 8 to 10 microns in diameter with a nucleus that is often round but may be irregular. The chromatin structure appears uniformly dense and dark with no parachromatin readily evident. It possesses an abundant amount of cytoplasm very similar in color to that of the mature red cell being bluish/buff to buff/pink.

All cells discussed thus far have a nucleus and are therefore sometimes referred to collectively as "NRBC's" or nucleated red blood cells. These cells are numerous in the bone marrow, but are not normally seen in the peripheral blood. They must be reported whenever seen in the peripheral blood, even in the newborn where up to 10 NRBC's/100 WBC's may be seen without being abnormal.

The metarubricyte, having lost its nucleus by extrusion, becomes what is known as a diffuse basophilic erythrocyte (reticulocyte); the first non-nucleated stage in the maturation sequence of the erythrocyte. This cell is 8 to 9 microns in diameter and stains a bluish/buff to buff/pink. As the reticulocyte matures the bluish color (which is due to ribonucleic acid and protoporphyrin characteristic of immature cells) decreases in intensity and may be very difficult to detect (hence the need for special supravital stains when specifically enumerating reticulocytes). A few reticulocytes are normally present in the peripheral blood, although they constitute a very small part (0.5 to 1.5 percent) of the total number of circulating red cells.

The mature erythrocyte has lost its cytoplasmic basophilia and is no longer regarded as a reticulocyte. The mature red cell is 7 to 8 microns (average 7.5 microns) in diameter and has the shape of a biconcave disc. Its color is buff pink, sometimes described as orange red or salmon pink, and its characteristically shows a slight central pallor, or lightly-colored central area, as a result of its biconcave shape.

Thus far normal erythrocyte morphology has been discussed. Abnormal erythrocyte morphology occurs and must be considered because it is often the indication of a serious illness.

Mature erythrocytes are normally 7 to 8 microns in diameter. Anisocytosis refers to a major variation in size (minor variations are considered normal). To be more specific, macrocytes are red cells that are 9 microns or in diameter. Macrocytosis is normally seen in the newborn and is characteristic of anemias resulting from a deficiency of either vitamin B_{12} or folic acid (e.g., pernicious anemia). Microcytes, on the other hand, are red cells that are 6 microns or less in diameter. Iron deficiency anemia is probably the most common condition in which microcytes are seen. Macrocytes and microcytes can be seen in a variety of other conditions as well as those given as examples.

Poikilocytosis is a general term that refers to any major variation in the shape of red blood cells. An example is the sickle-like cell of poikilocytosis which is an elongated red cell generally having rather pointed ends or projections. Sickle-like cells occur when red cells containing hemoglobin S are subjected to lowered oxygen tension. Hemoglobin S is an abnormal hemoglobin that is inherited in "sickle-cell anemia" (homozygous condition) and "sickle-cell trait" (heterozygous condition). The homozygous condition is debilitating and fatal, but patients with the trait generally lead normal lives.

Ovalocytes, (elliptocytes), also a form of poikilocytosis, are elongated red cells with blunt, rounded ends. Ovalocytosis is inherited as a Mendelian dominant. About 12 percent of the individuals with ovalocytosis develop a hemolytic anemia which varies from mild to severe in degree. Those who do not develop hemolytic anemia are usually asymptomatic. The number of ovalocytes present in the blood increases with age; the initial ovalocytes do not appear until about the third month after birth. The red cell precursors of the ovalocyte, NRBCs and reticulocytes, do not exhibit the oval shape. There is no reason to believe that ovalocytic hemolytic anemia is necessarily the result of being homozygous for the ovalocytosis gene.

Sperocytes are spherical red blood cells, usually about 4 microns in diameter; however, on rare occasions they may be as large as normal red cells. They are recognized by their size; lack of central pallor, since the cell is not a biconcave disc; and dark color. Spherocytosis is inherited as a Mendelian dominant, although some cases have been reported in which both parents were found to be hematologically normal. Hereditary sperocytosis is extremely variable in its degree of severity. Spherocytes can also be seen in other hemolytic anemias. Red cells stored for transfusion may become spherical over a period of time as a result of altered pH, effect of anticoagulant, ionic shifts, or by metabolic exhaustion. For this reason, spherocytes may also be seen in patients who have recently received a transfusion. When spherocytes are microcytic they are sometimes called "microspherocytes."

Crenated red cells are not clinically significant when seen on peripheral blood smears, but must be distinguished from other significant forms of poikilocytosis. Crenated red cells exhibit projections that are short, blunt, and fairly evenly spaced. Crenation occurs as a result of the blood having stood for a long time, or as a result of the red cells having been exposed to lytic agents or hypertonic solutions.

"Irregularly contracted cells" ("bizarre forms") are oddly shaped cells that do not fall into any of the specific categories of poikilocytosis already mentioned. Some of these cells probably represent special forms of poikilocytosis and have been given more specific names (e.g., burr cells, acanthocytes, and schistocytes). In addition, some of these "bizarre forms" are merely manifestations of a more specific form of poikilocytosis, for example, when sickle-like cells are present there are also usually present oddly-shaped cells that result from the sickling process itself. We are using the category of "irregularly contracted cells" or "bizarre forms," therefore, to collectively refer to any oddly shaped cells that do not fall in the category of the more specific descriptions mentioned.

Hypochromic cells are those that are deficient in hemoglobin. They may be recognized as red cells having an increased central pallor, sometimes to the extent that only a thin rim of hemoglobin is visible. They are an indication of anemia and are often microcytic, as in iron deficiency anemia.

Target cells are red cells that appear to have a central condensation of hemoglobin and therfore appear dark in the center and around the periphery with a lighter zone in between. Target cells are basically platycytes (flat cells) and their formation is probably an artifact caused by fixation and staining, since they do not appear in wet preparations. We have grouped them in the same category, hemoglobin variations, as hypochromic cells because they are commonly seen in diseases due to hemoglobin deficiencies or abnormalities as thalassemia, hemoglobin C disease and sickle-cell anemia.

Abnormalities considered as erythrocytic inclusions are polychromatic cells, basophilic stippling, and Howell-Jolly bodies. Polychromatic cells are red cells that have a bluish buff color when stained with Wright's stain. The bluis- color is due to the presence of basophilic substances (ribonucleic acids and protoporphyrins) characteristic of immature cells such as reticulocytes. Red cells exhibiting this bluish buff color should not be referred to as reticulocytes routinely, however, because special supravital stains are necessary to accurately enumerate reticulocytes that possess minimal amount of the basophilic substances. Polychromatic cells are seen when there is increased bone marrow activity in which immature red cells are being released into the circulation at an accelerated rate. Polychromatic cells are sometimes referred to as being diffusely basophilic cells (diffuse basophilia).

Basophilic stippling is the term used to describe cells that show aggregations of the basophilic substance in the form of a large number of coarse or fine blue granules. Basophilic stippling is often seen in lead poisoning, where the stippling reflects both an abnormality in ribosomal aggregates and iron-laden mitochondria.

Howell-Jolly bodies are thought to be nuclear remnants remaining in the cell after the metarubricyte extrudes its nucleus. This belief is supported by the fact that Howell-Jolly bodies are Feulgen-positive, indicating the presence of deoxyribonucleic acid, a component of the metarubricyte's nucleus.

Howell-Jolly bodies are frequently seen in pernicious anemia and following splenectomy. It is conjectured that one of the functions of the spleen is to remove erythrocytic inclusions. They are round, dark violet, and usually one micron or less in diameter. Generally, only one Howell-Jolly body will be found in any red cell. On rare occasions, two or more may be seen in a single cell.

Several things must be considered when evaluating erythrocyte morphology. First of all, this evaluation should be performed using the oil immersion objective of microscope in order not to miss any small, but nevertheless significant details. Second, the time expended doing the leukocyte differential count should be used efficiently by noting the condition of the erythrocytes while the count is being made. After the differential count has been completed, it is advisable to scan the slide further while concentrating on detecting any abnormalities that might be present. Only after this has been accomplished should a morphology report be rendered.

If nothing abnormal is noted with respect to erythrocyte morphology, a report of "N/N", meaning "Normocytic-normochromic," should be rendered. "Normocytic" indicates that the red cells are of normal size and shape, while "normochromic" indicates that the red cells are of normal color and hemoglobin content.

When abnormalities are noted, always use specific terminology in reporting them. In other words, never use broad and possibly ambiguous terms like "anisocytosis;" instead use more specific terms such as "macrocytosis" or microcytosis."

Nucleated red blood cells should be enumerated and reported as the number of NRBCs seen per 100 WBCs, the number of WBCs counted in a routine differential. This method of enumeration will make corrections of the white cell count easy to perform.

An attempt should be made to evaluate the degree of other abnormalities such as macrocytosis and ovalocytosis, as well. In doing this, it must be kept in mind that very small variations of size and shape occur even on normal smears. Care must be taken not to confuse these normal variations with true abnormalities. Abnormalities can be graded as "slight," "moderate," or "marked" in degree; some investigators prefer to use 1+, 2+, 3+ and 4+ instead. We will define a "slight" abnormality as one where abnormal forms are seen only intermittently on the slide. A "moderate" condition is one in which we see a few abnormal forms in most of the microscopic fields observed. "Marked" will be used to refer to the presence of several abnormal forms in virtually all fields observed. An exception to this manner of reporting concerns Howell-Jolly bodies -- they may be reported simply as "present" or "seen."

Always consult your local laboratory SOP for possible exceptions to these rules of evaluating morphology so that consistency of reporting may be maintained within the laboratory.

The origin and development of erythrocytes has been delineated. Several different erythrocyte abnormalities were explained. The manner by which thin blood smears should be examined to ascertain the morphology of red cells was explained, as was the manner in which both normal and abnormal morphology should be reported.

The evaluation of erythrocyte morphology is an integral part of every differential. However, the ability to evaluate morphology correctly is the result of a great deal of study and serious concentration at the microscope. Do not take this task lightly, since your power of observation relative to erythrocyte morphology may be of invaluable assistance to the physician and his patient.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-027
LABORATORY SCIENCES DIVISION 075
CLINICAL MICROSCOPY BRANCH

Miscellaneous Hematological Procedures (3 hours)

COURSES PRESENTED TO: 311-92B20 Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES:

TM 8-227-4

STUDY ASSIGNMENT: After class study TM 8-227-4, pp 4-18 thru 4-19, 5-10 thru 5-12, 5-14 thru 5-15 and 5-20 thru 5-22.

STUDENT UNIFORM AND EQUIPMENT: None

TOOLS, EQUIPMENT, AND MATERIALS: None

PERSONNEL: One primary instructor (MOS 92B4H, 92B3H or 92B2H)

INSTRUCTIONAL AIDS: PA system with lapel microphone, chalk, chalkboard and eraser

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

I. INTRODUCTION (5 min)

A. Opening Statement: What is a "Special Hematological Procedure?" As the name implies, it is a test which gives the physician specialized information on specific functions of the body: i.e., the reticulocyte count is an index of erythropoiesis; the cerebrospinal fluid cell count is a tool in diagnosis of brain and central nervous system disease or damage, and the sickle cell preparation is a screening test for sickle cell disease. These tests are not performed on a rountine day to day basis on each and every patient as is the complete blood count. They are administered only when required to supply specific data on the patient's condition. We will discuss each examination in turn, looking at the use of each of these special procedures and their role in diagnosis.

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B. Objectives

1. List and explain the steps in performing the reticulocyte count, and interpret the results of testing.

2. Outline the procedure for performing cerebrospinal fluid examinations, with emphasis on normal values and calculations.

3. Discuss the two forms of sickle cell disease, with emphasis on severity and laboratory findings.

4. Outline the sickle cell preparation procedure and explain the results of testing.

C. Class Procedure and Lesson Tie-in

1. Two hours of lecture on miscellaneous hematological procedures.

2. Practical exercise in accordance with individual needs.

3. Conference as necessary to solve individual problems.

II. **EXPLANATION** (80 min)

A. Reticulocyte Count

QUESTION: What is a reticulocyte?

ANSWER: A reticulocyte is an erythrocytic cell in the state of development between the nucleated forms and the mature erythrocyte.

1. Erythrocyte maturation review

NOTE: The level of reticulocytes is proportional to bone marrow activity.

2. Significance of altered reticulocyte count

   a. Increased values

   b. Decreased values

3. Description of reticulocytes

   a. Size: 8 to 9 microns

   b. Color: darker buff color

   c. Reticulum: network (basophilic substance)

QUESTION: Are reticulocytes indentifiable in Wright's stained blood smears?

ANSWER: No, the reticulum is visible and identifiable only when stained with a supravital stain.

4. Principle

5. Equipment

   a. Slides

   b. Capillary tubes

   c. Test tubes

6. Reagents

   a. Supravital stains

      (1) Brilliant cresyl blue

      (2) New methylene blue

   b. Counter stain: Wright's stain (optional)

7. Procedure

   a. Place one (1) capillary tube of blood in test tube

   b. Add equal volume of supravital stain

   c. Mix blood and stain; allow to stand for 5 to 10 minutes for staining to occur

   d. Prepare thin blood smear from stained erythrocytes

   e. Counter stain with Wright's stain (if desired)

   f. Under oil immersion, count the number of reticulocytes in five (5) fields of 200 erythrocytes (1000 RBC's)

   g. Calculations: $\frac{\text{\# retics/1000 RBC's}}{10}$ = percent reticulocytes

8. Sources of error

   a. Precipitated stain

   b. Supravital stain prepared with alcohol (cell fixation)

   c. Non-viable (old) specimen (1/2 hour)

  9. Normal values

   a. Birth 2.6 to 6.0 percent

   b. Adult 0.5 to 1.5 percent

B. Cerebrospinal Fluid Cell Count

  1. Source and Collection

   a. Obtained by physician (spinal tap)

   b. Routing of tubes

    (1) Tube number 1 to bacteriology

    (2) Tube number 2 to chemistry

    (3) Tube number 3 to hematology

  2. Precautions

   a. Infection (hazard)

   b. Cell disintegration (STAT procedure)

  3. Equipment

   a. Bactericidal solution (phenol or iodine)

   b. Capillary tubes

   c. WBC pipets

   d. Hemacytometer and coverslip

  4. Reagent: cerebrospinal fluid diluent

   a. Acetic acid 10 percent

   b. Crystal violet

5. Preliminary examination: color and appearance
    a. Normal
        (1) Clear
        (2) Colorless
    b. Abnormal
        (1) Turbid (cloudy - infection)
        (2) Red (bloody - RBC's present)
        (3) Xanthochromic (yellow - decomposed blood pigments)
6. Total cell count: undiluted count
    a. Principle (enumeration of all cellular elements)
    b. Procedure
        (1) Fill hemacytometer with well-mixed, undiluted spinal fluid from a capillary tube and allow cells to settle for 5 minutes
        (2) Examine entire ruled are (9 sq. mm.) for cells

NOTE: If cell count is high, dilution with Hayem's solution may be indicated

        (3) Note condition of erythrocytes
            (a) Crenated
            (b) Fresh
        (4) Calculations

$$\frac{\#\text{cells} \times 10\ (\text{depth}) \times 1\ (\text{dilution})}{9\ (\text{area})} = \#\ \text{cells} \times 1.1$$

7. Leukocyte count: diluted count
    a. Principle
        (1) Lysis of red cells (by acid)
        (2) Staining of leukocytes (by crystal violet)

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b. Procedure

(1) Draw CSF diluent to 1.0 mark

(2) Fill to 11 mark with cerebrospinal fluid

(3) Mix (by hand, with figure-eight motion) for two minutes

(4) Fill hemacytometer and allow to settle for 5 minutes

(5) Examine entire ruled area (9 sq. mm.) for cells

(6) Calculations

$$\frac{\#\text{ cells} \times 10 \text{ (depth} \times 10/9 \text{ (dilution)}}{9 \text{ (area)}} = \#\text{ cells} \times 1.2$$

8. Differential Cell count

a. Under 100 cells/cu. mm.: perform rough differential on the hemacytometer, using the high dry objective

b. Over 100 cells/cu. mm.

(1) Centrifuge remaining specimen for cell button

(2) Prepare thin smear with cell button, and stain with Wright's stain.

(3) Perform differential cell count in the usual manner

9. Erythrocyte count calculation

Total cell count (-) leukocyte count (=) erythrocyte count

10. Normal values

a. Erythrocytes	0 erythrocytes/cu. mm.
b. Leukocytes	0-5 cells/cu. mm. (chiefly lymphocytes)

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NOTE: The occurrency of any neutrophils is pathological.

C. Sickle Cell Preparation

1. Sickle cell phenomenon

    a. Cause: hemoglobin-S: (results in loss of normal erythrocyti
    shape when subjected to reduced oxygen tension)

    b. Clinical significance and genetic transmission

        (1) Sickle cell trait

            (a) Heterozygous: one hemoglobin-S gene

            (b) Prognosis: "benign" condition

            (c) Laboratory findings

                1 Screening test: positive

                2 Electrophoresis: percent identification of hemoglobin-S

                3 Peripheral blood: normal except in crisis

        (2) Sickle cell anemia

            (a) Homozygous: both hemoglobin-S genes

            (b) Prognosis: symptomatic disease condition with shortened lifespan

            (c) Laboratory findings

                1 Screening test: positive

                2 Electrophoresis: percent identification of hemoglobin-S

                3 Peripheral blood: generally anemic; poikilocyto
                sickle-like cells; increased metarubricytes

2. Sickle cell preparation

    a. Principle

        (1) Oxygen tension reduced (*in vitro*)

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(2) Microscopic examination (of erythrocytes)

b. Equipment

(1) Slides

(2) Coverslips

(3) Droppers

(4) Applicators

c. Reagent: fresh 2 percent solution of sodium metabisulfite

d. Procedure

(1) Place one drop of blood on clean glass slide

(2) Add two drops of reagent

QUESTION: What is the function of the sodium metabisulfite?

ANSWER: It acts to lower the oxygen tension in the specimen, and induces sickling to occur if hemoglobin-S is present in the erythrocytes.

(3) Mix with applicator and apply coverslip

(4) Express excess blood suspension

(5) Read microscopically

(a) Immediately

(b) 15 minutes

(c) 30 minutes

(6) Interpretation of results

(a) Negative: no sickling

(b) Positive: sickling (percent and time)

e. Sources of error

(1) Sodium metabisulfite solution not fresh

(2) Presence of abnormal hemoglobin other than hemoglobin-S

3. Commerical test kits: macroscopic determination

4. Hemoglobin electrophoresis: definitive identification of hemoglobin protein molecules

D. Questions from Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Reticulocyte count

2. Cerebrospinal fluid cell count

3. Sickle cell preparation

B. Closing Statement: We have discussed three specialized tests that are seldom performed on a "routine" basis in the clinical laboratory. Each of these tests supplies the physician with specific information necessary for the diagnosis and treatment of his patients.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *IM 08-280-027
LABORATORY SCIENCES DIVISION 075
CLINICAL MICROSCOPY BRANCH

Miscellaneous Hematological Procedures

What is a "Special Hematological Procedure?" As the name implies, it is a test which gives the physician specialized information on specific functions of the body: i.e., the reticulocyte count is an index of erythropoiesis; the cerebrospinal fluid cell count is a tool in diagnosis of brain and central nervous system disease or damage; and the sickle cell preparation is a screening test for sickle cell disease. These tests are not performed on a routine day to day basis on each and every patient as is the complete blood count. They are administered only when required to supply specific data on the patient's condition. We will discuss each examination in turn, looking at the use of each of these special procedures and their role in diagnosis.

The reticulocyte count is an indicator of the amount of erythrocytes the bone marrow is releasing into the peripheral blood. By monitoring the reticulocyte level the physician may evaluate the course and treatment of the anemic patient. These data enable the physician to determine whether or not the patient's marrow is producing an adequate number of erythrocytes, and adjust treatment accordingly.

In previous lectures we have studied the function and maturation of the erythrocytes. You will recall that the immature erythrocytes are produced in the bone marrow where they remain until they approach maturity.

Development begins with the rubriblast, proceeding to the prorubricyte, rubricyte and finally the metarubricyte, halving nuclear volume in each of the four stages. These four nucleated phases of development are normally confined to the bone marrow; their presence in peripheral blood indicates an abnormal condition. When the nucleus of the metarubricyte is ejected, the cell becomes a reticulocyte which in turn matures to become an erythrocyte. This maturation sequence is relatively rapid because of normal replacement demands placed on the hematopoetic system due to the limited lifespan (120 days) of the mature red corpuscles.

By observation and studies on bone marrow and peripheral blood, reticulocytes are demonstrable in peripheral blood within four to six days after formation of a rubriblast. This production of reticulocytes may be due to normal body stimuli to replace red corpuscles after their normal lifespan, or in response to other stimuli.

An altered reticulocyte count is indicative of changes in erythropoesis. Reticulocytosis indicates increased erythrocyte production. Acute hemorrhage is usually followed by accelerated erythrocyte production indicated by a reticuloyctosis. The increased reticulocyte count is expected by the third or fourth day in the usual case, but may occur earlier in massive hemorrhage. Chronic hemorrhage is also followed by increased erythropoesis, though the

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reticulocytosis is not as great as in acute hemorrhage. In some types of untreated chronic anemia the reticulocyte count is depressed. When therapeutic drugs are administered to the patient to stimulate the hematopoetic system, there is usually a dramatic increase in the reticulocyte count.

After the metarubricyte expel its nucleus, a small amount of basophilic substance characteristic of immaturity remains in the cytoplasm. This vestige of cellular maturation is composed chiefly of ribonucleic acid and is distributed evenly throughout the cell. In a Wright's-stained smear the reticulocyte appears somewhat larger (eight to nine microns) than mature erythrocytes. The reticulocytes may stain a darker buff color with Wright's stain due to the presence of the basophilic substance. After fixation and staining the basophilic substance may be evenly distributed throughout an erythrocyte (diffuse basophilia), or the cell may have a mottled appearance with orange and blue areas (polychromatophilia).

When stained with a supravital stain the reticulum of basophilic substance is easily demonstrated. The most familiar form taken by the reticulum is a filamented aggregate which become granular as the cell matures. The reticulum may be abundant or sparse, according to the stage of development, and may appear coarse or fine depending on pH and the supravital stain used.

It must be noted that reticulocytes are not positively identifiable in Wright-stained preparations. A supravital stain is a dye of low toxicity that will react with the nuclear remnants and without causing the cell to die as occurs in routine Wright-staining techniques. (The methanol in Wright's stain dehydrates and fixes all cellular elements.) The reticulum is stained with a supravital stain such as brilliant cresyl blue, rendering the nuclear remnants visible and the reticulocyte identifiable.

Brilliant cresyl blue is the most commonly employed supravital stain for reticulocyte enumeration. Brilliant cresyl blue is mixed with the anticoagulant sodium citrate and dissolved in isotonic saline. Other stains may be employed to demonstrate the reticulum, e. g., Nile blue sulfate or new methylene blue.

Some laboratories find it desirable to counterstain reticulocyte preparations with Wright's stain. (NOTE: Counterstaining must be accomplished after supravital staining is complete to avoid fixation before demonstration of the reticulum.) The Wright's stain dyes the erythrocytes their customary buff color, thus adding contrast to the blue reticulum of the reticulocytes in the preparation. Counterstained reticulocyte preparations may be employed for differential cell counts without the need to prepare a separate thin blood smear.

The technique for reticulocyte staining is outlined as follows:

1. Measure one volume of fresh blood using a capillary tube filled

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to capacity. Transfer the blood to a clean, previously labeled test tube. Blood for the reticulocyte enumeration may be obtained by either venipuncture or finger puncture.

2. Add an equal volume of supravital stain to the tube.

3. Mix the blood and stain; place tube in a test tube rack and allow the mixture to stand for five to ten minutes. During this time the dye penetrates the red cell and combines with the nuclear vestiges.

4. Prepare a thin blood smear using the blood and stain mixture.

5. Counterstaining may be accomplished at this point, at the option of the technician performing the count.

6. The procedure is accomplished by counting the number of reticulocytes occurring per 1000 erythrocytes. The oil immersion objective of the microscope must be utilized to identify the stained reticulum characteristic of reticulocytes. The reticulocytes appear as erythrocytes containing refractile blue threads or granules. Erythrocytes with blue filaments are young reticulocytes, and those with granules are older forms of reticulocytes. Enumeration may be accomplished more rapidly by counting the number of reticulocytes occurring in five fields of 200 erythrocytes. (200 erythrocytes per oil immersion field are normally encountered in the thin portion of the smear suitable for differential cell counts. The accuracy of the 200 cells per field may be confirmed by actually counting the number of erythrocytes in one quarter of each field.)

7. Reticulocytes are reported as the number of reticulocytes per 100 erythrocytes; that is, as the percent of mature erythrocytes. Calculation is based on the following formula:

$$\frac{\text{\# retics counted/1000 RBC's}}{10} = \text{percent reticulocytes}$$

EXAMPLE: A total of 27 reticulocytes are encountered in five fields of 200 cells.

$$\frac{27}{10} = 2.7 \text{ percent reticulocytes}$$

The most common problems encountered during the performance of reticulocyte counts is precipitated stain. The precipitated stain may be confused with reticulocyte granulation by less experienced technicians. Confusion may be minimized by remembering that the stain precipitate is not refractile and will be observed throughout the smear; while reticulum is refractile and confined within the red cell.

Precipitated stain may be avoided by filtering the stain prior to use. This precaution applies to both the supravital stain and to the optional counterstain.

The supravital stain must be prepared according to formula. If an alcoholic solution of dye is prepared it will not be effective in staining reticulocytes. The alcohol fixes the cell, interfering with the absorption of the supravital stain by the cell's reticulum.

Reticulocyte counts should be stained and the thin smears prepared within a half hour of collecting the venous blood specimen to avoid erroneously low results. The reticulocyte continues to mature in vitro after collection. Once the smear is prepared the slide may be set aside for enumeration at a later time.

Under normal conditions we should find one reticulocyte present in the bone marrow for each nucleated red cell. In peripheral blood reticulocyte values represent 2.6 to 6.0 percent of the erythrocytes at birth, decreasing by the second week post partum to adult values. In adults the reticulocytes normally represent 0.5 to 1.5 percent of the red cells in peripheral circulation.

The examination of cerebrospinal fluid is always an emergency procedure requiring utmost speed and care. The cell count must be performed on a STAT basis to preclude reporting erroneous information to the physician. Spinal fluid specimens must be handled with extreme caution to avoid contamination of the laboratory.

The cerebrospinal fluid specimen is obtained by the patient's physician. A long needle is introduced between the vertebrae in the lower back into the meninges in the lumbar region and the specimen withdrawn. The spinal fluid is placed into three sterile tubes labeled 1, 2, and 3 (in reverse order). The hematology section usually receives all three tubes of spinal fluid. The individual receiving the spinal fluid specimens has the responsibility of seeing that the specimen tubes are distributed to the proper section of the laboratory for analysis. Tube number one is most likely to contain extraneous material (e.g., blood cells from trauma of collection, and microorganisms) so it is sent to bacteriology for culture and other examinations. Tube number two is sent to chemistry for glucose and total protein. Tube number three is analyzed by hematology since it is most likely to be free of cellular material introduced by the trauma of collection. The third tube is more likely to be representative of the spinal fluid's cell content than the other two tubes.

EXTREME CAUTION MUST BE USED WHENEVER HANDLING CEREBROSPINAL FLUID SPECIMENS to insure that any infectious material does not endanger laboratory personnel. Cerebrospinal fluid is an ideal cultural medium for most pathological micro-organisms affecting man; therefore, extreme care must be used to prevent spreading disease (e.g., tuberculosis, poliomyelitis, syphilis, and meningitis).

Performing cell counts on cerebrospinal fluid must always be performed on a STAT basis so that examination is completed within thirty minutes after the specimen is withdrawn from the patient because of continued growth of any organisms present in the specimen. In addition, the red corpuscles will deteriorate upon standing with continued crenation and/or hemolysis.

Most laboratories have specifically designated equipment for performing cerebrospinal fluid examinations because of the contamination hazard. There should be a container of bactericidal solution, such as phenol or iodine, into which all equipment coming into direct contact with the specimens is placed. The bactericidal solution must be of sufficient concentration to kill all pathogens on contact. Equipment should be removed from the solution and cleaned in accordance with the laboratory SOP. A special, separate area usually designated to minimize contamination hazards should any of the spinal fluid be accidentally spilled in the process of examination. This special area is also maintained in accordance with the laboratory SOP, and must be cleaned with the bactericide before and after each specimen is processed.

Plain capillary tubes may be employed to transfer undiluted cerebrospinal fluid from the specimen container to the hemacytometer; however, Thoma pipets are required to make necessary dilutions. A supply of hemacytometers adequate for anticipated work load should be designated only for spinal fluid use.

Cerebrospinal fluid diluent is used for routine dilution of spinal fluid for leukocyte enumeration and rough differential cell count. The diluent is an aqueous solution of 10 percent acetic acid with crystal violet. The increased concentration of acetic acid (as compared to WBC diluent) is necessary to hemolyze the erythrocytes due to the reduced dilution of the spinal fluid in routine counts. The crystal violet stains the leukocytes for rough differential counts.

As soon as the cerebrospinal fluid is received in the laboratory a preliminary examination is performed, i.e., the color and appearance of the specimen is noted. Under normal conditions the spinal fluid specimen will be clear and colorless. Any color or cloudiness is indicative of an abnormal condition.

Turbidity (cloudiness) is symptomatic of bacterial contamination, with or without accompanying leukocytosis. Meningeal infection is marked by turbidity, with increased cloudiness indicating a more severe infection. If the leukocyte count is increased over 500 cells per cubic millimeter the specimen is inevitably turbid.

A red specimen indicates the presence of erythrocytes, either from intracranial hemorrhage or from the trauma at the site of specimen collection. If frank blood with spontaneous clotting is observed, a bloody spinal tap is indicated. Microscopic examination is helpful in determining the location of hemorrhage and elapsed time since the bleeding occurred.

Xanthochromia develops after intracranial hemorrhage has been present for a few hours. The yellowish color is due to the disintegration of blood pigments and the breakdown of hemoglobin. Xanthochromia may develop following subarachnoid hemorrhage, from tumors, abscesses and inflammation.

A total cell count is performed on undiluted cerebrospinal fluid for enumeration of all cellular elements present in the specimen. The procedure is outlined as follows:

1. With a plain capillary tube, fill one hemacytometer chamber with well-mixed, undiluted spinal fluid. Allow the cells to settle for five minutes.
2. Examine the entire ruled area of the hemacytometer (9 sq. mm.) under the low power objective for the presence of any cells. (Note: if the cell count is high (over 500 cells per cu. mm.) dilution with Hayem's solution is indicated.)
3. If erythrocytes are seen, note their condition. Fresh cells are seen in bloody spinal taps and active intracranial bleeding. Crenated cells are noted in recent intracranial bleeding and in specimens which were obtained more than thirty minutes before examination.
4. Calculation of total cell count is completed using the following formula:

$$\frac{\#\text{ cells counted} \times 10 \text{ (depth)} \times 1 \text{ (dilution)}}{9 \text{ (area)}} = \text{cells/cu. mm.}$$

It should be noted that the dilution factor is one because the specimen has not been diluted. The constant for calculation is 1.1 (times the number of cells counted).

The leukocyte count is performed on spinal fluid using the cerebrospinal fluid diluent if any cellular elements are observed in the performance of the total cell count. Cells exposed to spinal fluid may be distorted and almost unrecognizable under the microscope, therefore all specimens must be diluted to preclude any errors in cell identification. The acetic acid effectively hemolyzes all erythrocytes, and the crystal violet stains the leukocytes for positive identification. The procedure for performing the diluted leukocyte spinal fluid cell count is as outlined below:

1. Using a WBC pipet draw diluent to the 1.0 mark.

2. Draw well-mixed spinal fluid to the 11 mark of the pipet. It should be noted that spinal fluid dilutions are a reversal of the techniques employed for diluting blood specimens.

3. Shake the pipet by hand using the figure-eight motion for two full minutes. The use of mechanical shakers is discouraged since the shakers tend to accelerate the rate of cellular disintegration.

4. Discard the fluid in the capillary portion of the pipet into the bactericidal solution, and fill the hemacytometer.

5. After allowing the cells to settle for five minutes, count all leukocytes on the hemacytometer grid, using the low power objective.

6. Calculation of the leukocyte count is made according to the following formula:

$$\frac{\#\text{ cells counted} \times 10 \text{ (depth)} \times 1.1 \text{ (dilution)}}{9 \text{ (area)}} = \text{cells/cu. mm.}$$

It must be noted that in routine spinal fluid dilutions the diluent is drawn to the 1.0 mark and the pipet is filled to the 11 mark with the cerebrospinal fluid specimen; therefore, the dilution factor is 10/9 or 1.1. The constant for calculation of the leukocyte count is 1.2 (times the number of cells counted).

A differential count should be performed whenever leukocytes are present in the spinal fluid. A rough differential is performed on the hemacytometer if the cell count is below 100 leukocytes per cubic millimeter; for counts over 100 leukocytes per cubic millimeter a smear must be prepared for performance of the differential count.

Crystal violet is absorbed by the leukocytes as a monochromatic stain, permitting differentation of cellular morphology when using the cerebrospinal fluid diluent. Leukocytes are categorized by their nuclear structure: cells which are segmented are "polys" (neutrophils), and cells without separate lobes are "lymphs" (including all forms of mononuclear leukocytes). Further differentation of leukocytes is unnecessary when performing rough differential cell counts on cerebrospinal fluid on the hemacytometer.

The rough differential count is performed by characterizing all leukocytes in the ruled area of the hemacytometer as "lymphs" or "polys" with the high dry power objective. The results are reported as the actual number of each type of cell identified. (E.g., if 10 cells are actually counted and differentiated as 3 "polys" and 7 "lymphs," these values are reported, along with the leukocyte count of 12 leukocytes per cubic millimeter.)

When the leukocyte count is over 100 leukocytes per cubic millimeter, the differential count is performed by preparing a smear from the specimen. The procedure is outlined as follows:

1. Centrifuge the remaining spinal fluid specimen to obtain a cell button.

2. Decant the supernatant fluid and resuspend the cell button with the fluid remaining in the specimen tube. Prepare a thin smear from the cell button and allow it to air dry. (Note: It may be necessary to use egg albumin or cell-free serum to make the sediment adhere to the slide.)

3. Perform the differential cell count in the usual manner using the oil immersion objective. The results are reported as the percent occurrence of each type of leukocyte.

The erythrocyte count may be calculated by subtracting the leukocyte count from the total cell count. The erythrocyte count may be of value to the physician in ascertaining the degree of intracranial bleeding.

Cerebrospinal fluid does not normally contain any erythrocytes. (However, erythrocytes are encountered in many specimens because of the trauma of obtaining the specimen by spinal tap.) Adults normally have between 0 and 5 leukocytes per cubic millimeter in spinal fluid. All leukocytes encountered in cerebrospinal fluid are normally lymphocytes. Any "polys" are considered abnormal and must be reported to the physician.

Sickle cell anemia has come to national attention since President Nixon's health message of 1971, during which he pledged national priority for a cure of sickle cell anemia. As many as 2,500,000 American blacks have sickle cell disease, of which one in forty whose erythrocytes "sickle" are victims of a chronic and severe hemolytic type of anemia ..... "sickle cell anemia."

By way of review, we recall that hemoglobin is the major component of the erythrocyte. Hemoglobin transports oxygen from the lungs to the body's tissues and along with plasma transports carbon dioxide from the tissues to the lungs.

The hemoglobin molecule is a conjugated protein (i.e., the globin portion) and four heme molecules, each of which contain one molecule of iron in the ferrous (Fe^{++}) state. This ferro-heme is a metallic compound with an iron atom at the center of a porphyrin ring; it serves as the active portion of the hemoglobin molecule, combining with one molecule of oxygen (or carbon dioxide) in a reversible manner, and imparts the red color to the red corpuscle.

The basic protein, globulin, is composed of two pairs of polypeptide chains which are designated as alpha chains and beta chains. Each polypeptide chain is made up of about 150 amino acids in specific order. The normal adult hemoglobin has been designated as hemoglobin-A. In sickle cell disease, the normal sequence of amino acids has been altered by a genetically controlled substitution with valine replacing glutamic acid in the sixth position from the "N" terminal in the beta chain. This abnormal form of hemoglobin has been designated hemoglobin-S.

There are several theories explaining why erythrocytes containing hemoglobin-S tend to lose their normal biconcave discoidal shape and assume "sickle" forms when subjected to reduced oxygen tension. It is known that sickling is a result of an intracellular interaction of the abnormal hemoglobin-S molecules. The most widely accepted current theory of the precise mechanism is that of Murayama. Murayama hypothesizes that when oxygen tension is lowered, hydrophobic bonding between adjacent hemoblobin molecules takes place as a result of conformational changes in the hemoglobin-S molecules. This bonding, which does not occur between hemoglobin-A molecules, causes the hemoglobin-S molecules to lock together and exert force on the erythrocyte's cell membrane. This pressure results in the changes in the shape of the cell. Erythrocytes which have lost their usual discoidal structure (i.e., sickled) are less able to carry oxygen which results in a definite reduced oxygen carrying capacity. There appears to be two types of sickled erythrocytes. The first type exhibits a filamentous process which may revert to discoidal shape on exposure to atmospheric oxygen tension; the second type, which is sickle shaped, fails to revert to discoidal form under these conditions.

There is an increase in mechanical fragility in sickle cell disease, and it has been proposed that intravascular hemolysis may be due to this abnormal mechanical fragility. There is also a demonstrable increase in the rate at which sickled erythrocytes are removed from the circulation by the body, a condition which may contribute to anemia.

Any condition resulting in restructured (sickled) erythrocytes, reduced oxygen carrying capacity OR increased erythrocyte destruction would be serious in and of itself; however, it appears that these conditions are at least partly dependent upon and compound the effect of one another ....resulting in sickle cell crisis. That is, if the oxygen tension within the body is reduced, changes in the hemoglobin-S molecule will cause sickling in the erythrocytes. The sickled erythrocytes become entangled causing an increase in blood viscosity with circulatory occlusion. The concentrations of carbon dioxide are increased from cellular metabolism (with consequent reduction of blood pH). These changes adversely affect the oxygen carrying capacity of the blood. This reduced oxygen carrying capacity results in a further reduction of oxygen tension, and the crisis cycle is continued.

The presence of this type of hemoglobin (i.e., abnormal hemoglobin-S) is an inherited characteristic. Hemoglobin-S is usually restricted to, but not exclusive in, the negroid populations. The characteristic is neither dominant nor recessive, but rather an autozomal or intermediate characteristic; that is, each gene controls the production of hemoglobin of only the type genetically encoded. If a single hemoglobin-S gene is inherited, approximately fifty percent of the hemoglobin produced will be abnormal hemoglobin-S. If the hemoglobin-S trait is inherited from both parents, most of the hemoglobin produced will be abnormal hemoglobin-S (the remainder of the hemoglobin produced will be fetal hemoglobin).

Approximately one in ten american blacks has the heterozygous condition termed sickle cell trait where the individual has inherited only one gene for the production of hemoglobin-S. Individuals with sickle cell trait are as a rule asymptomatic and the condition is benign. By this we mean individuals with sickle cell trait usually show no clinical symptoms of the condition, and may anticipate a normal lifespan. They may, however, have a "crisis period" brought on by severe physical stress (e.g., exposure to high altitude as on military aircraft operations or by extreme physical exertion) where cells containing hemoglobin-S are sickled as a result of lowered oxygen tension. The crisis period is usually short in duration and recovery is rapid.

The various screening tests for sickle cell disease are positive in the individual with sickle cell trait. Electrophoresis demonstrates twenty-two to forty-five percent hemoglobin-S, with the remainder of the hemoglobin being normal hemoglobin-A. The peripheral blood examination is generally unremarkable.

The homozygous condition, known as sickle cell anemia, is a serious chronic hemolytic anemia which affects one in forty of individuals with hemoglobin-S. Sickle cell anemia is a fatal condition since individuals with this anemia usually die in the first decade of life and few survive the fourth. These patients usually demonstrate pallor, weakness, jaundice, acute attacks of pain (especially in the abdomen), rheumatoid symptoms, bone changes and leg ulcers. The cause of death is usually not due to the severe anemia, but results from concurrent infection, renal failure or heart failure which follows a crisis.

The various screening tests for sickle cell disease are positive in patients with sickle cell anemia. Electrophoresis reveals seventy-six to one hundred percent hemoglobin-S, the remainder being fetal hemoglobin. Other laboratory findings may include: (1) erythrocyte count of one to two million cells per cubic millimeter (2) decreased erythrocyte sedimentation rate (3) peripheral blood smears with nucleated erythrocytes, (usually metarubricytes) polychromatophillia, basophillic stippling, occasional target cell, and "sickle-like cells" (poikilocytosis). No method of preventing the production of hemoglobin-S has been discovered since it is an inherited molecular disorder. Treatment is usually for secondary conditions and symptoms.

In the clinical laboratory, we may demonstrate sickle cells by performing the sickle cell preparation (metabisulfite method of Deland and DeSilva) and other specific tests. The sickle cell preparation is a screening test to determine if the erythrocytes will sickle when exposed to lowered oxygen tension. Bear in mind that only cells containing hemoglobin-S will assume the sickled forms. The degree of sickling and the severity of the disease show a positive correlation.

The oxygen tension is reduced in blood suspected of containing hemoglobin-S by mixing the specimen with a fresh solution of sodium metabisulfite. The metabisulfite. The metabisulfite acts as a reducing agent, lowering the oxygen tension in the specimen. The suspension is then observed microscopically for sickling.

Glass slides and coverslips are required for the microscopic examination of the wet smear when performing the sickle cell preparation. Medicine droppers are utilized for measuring and transferring the blood specimen and the reagent solution. Applicators facilitate mixing the blood and reagent on the glass slide.

Sodium metabisulfite ($Na_2S_2O_5$) is the only reagent required for this procedure. It must be noted that the metabisulfite solution is not stable; therefore, the solution must be prepared fresh daily to prevent erroneous results caused by deterioration of the reagent. A two percent solution of sodium metabisulfite is prepared using reagent grade metabisulfite.

The technique is outlined as follows:

1. Place one drop of the patient's whole blood on a clean, dry glass slide, utilizing either capillary or venous blood. If venous blood is used, the preparation should be made as soon after collection as practical.

2. Add two drops of the reagent solution to the glass slide.

3. Using an applicator, mix the blood and reagent until an even suspension is obtained. Place the coverslip over the top of the suspension to seal the mixture. (Note: It is not necessary to seal the edges of the preparation by rimming the coverslip with petrolatum.

4. Express the excess blood suspension by applying gentle pressure to the coverslip. This produces a film of blood thin enough to permit examination of individual erythrocytes under the microscope.

5. Place the preparation on the microscope stage and scan the slide looking for signs of sickle cell formation. The preparation should be read again at fifteen and thirty minute intervals from the time it was made. The two delayed readings are necessary to insure that the reducing agent has had sufficient time to lower the oxygen tension in the suspension.

If sickling is noted the sickle cell preparation is reported as positive. The speed and relative degree of sickling may not be used to differentiate the two disease forms. Confirmation of positive sickle cell preparations are normally made by the reference laboratory employing electrophoresis for content and concentration of types of hemoglobin present in the specimen. When sickling forms are not present, the test is reported as negative. (NOTE: The exact method of reporting sickle cell preparations varies from one laboratory to the next and will not be discussed here.)

The "rubber band test" (method of Scriver and Wagh) is a technique which may be utilized to screen for sickle cell disease when a suitable reducing agent is not available. A rubber band is placed around the proximal portion of the finger so as to restrict blood flow; the oxygen tension will be lowered by the continuing gaseous exchange with the metabolizing cells in the distal portion of the finger. After the restriction has been in place for five minutes, a capillary puncture is performed and a drop of blood is removed and sealed under a coverslip. The coverslip must be sealed by rimming with petrolatum around all edges to prevent gaseous exchange between the erythrocytes and the atmosphere.

The rubber band test is performed in the same manner as the sickle cell preparation with the exceptions outlined above. It should be noted that the rubber band test is rarely positive in cases of sickle cell trait due to the relatively high concentration of normal hemoglobin-A within the patient's erythrocytes.

There are several commercial sickle cell screening test kits available which rely upon the principle of the solubility test for hemoglobin-S detection. The principle advantage of the commercial test kits is reading results by macroscopic observation for turbidity which less skilled personnel should interpret correctly.

The test kits contain all materials, equipment and directions for testing including manufacturer's directions (which should be followed exactly).

Hemoglobin electrophoresis is a definitive test for determining concentrations and identification of several abnormal types of hemoglobin (including hemoglobin-S). When the hemoglobin proteins are placed on a surface in an electrolytic solution, and an electric current is applied, the molecules will migrate at varying specific rates according to their molecular structure. When compared with known standards, the identity and concentration of each type of hemoglobin in an unknown blood specimen may readily be made.

We have discussed three specialized tests that are seldom performed on a "routine" basis in the clinical laboratory. Each of these tests supplies the physician with specific information necessary for the diagnosis and treatment of his patients.

SFC Wilburn
*LP 08-280-0
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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
CLINICAL MICROSCOPY BRANCH

Hematological Techniques (73 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES: TM 8-227-4

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT AND MATERIALS: None

PERSONNEL: One primary instructor (MOS 92B3H or 92B4H); two secondary instructors (MOS 92B3H or 92B4H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, and eraser

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Practical exercise

I. INTRODUCTION (5 min)

A. Opening Statement: Your ability as a medical laboratory specialist will depend to a great extent on proficiency with your hands. A complete understanding of the theory and principles of laboratory procedures are of little or no value to you without the ability to carry out the actual test procedures. This 73 hour block of instruction will give you the basic manual skills to perform the routine hematological examinations with the high degree of accuracy necessary for successful completion of this course.

B. Objectives

1. Select and prepare specimens, reagents and controls necessary to perform routine hematological procedures.

2. Perform tests and procedures, interpret results, resolve discrepancies and accurately record and classify results.

C. Class Procedure and Lesson Tie-in

1. 73 hours of 128 hours hematology

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*This lesson plan supersedes LP 08-280-013, 014

2. Conference, as necessary, to solve individual problems

II. APPLICATION: (2905 min)

A. Arrangement of Students

1. Students will work at their regularly assigned area during the practical exercise.

2. Students will work in pairs when the practical exercise requires a venipuncture, or capillary puncture.

B. Instructions to Students

1. Obtain unknown blood specimens and/or blood films from instructor

2. Receive special equipment and/or reagents for exercise from instructor

3. Use equipment and reagents at students desks for completion of hematological procedures

4. Pick up microscope and transformer from cabinets as necessary for completion of hematological procedures.

5. Check chalkboard in classroom at the beginning of each period for special instructions.

6. Request assistance from instructor when needed

7. Report results of testing on report forms provided by instructor

C. Instructor's Activities

1. Prior to class

   a. Prepare reagents, as needed.

   b. Prepare and key unknown specimens for student analysis.

   c. Prepare and set up demonstrations as necessary.

2. During class

   a. The primary instructor will assign one instructor per table to observe and assist students.

b. Issue materials, reagents and specimens as necessary.

c. Monitor student's performance of procedures closely, making immediate corrections and answering student questions.

d. Check completed report forms and discuss problem areas with students.

e. Maintain classroom control.

D. Student's Activities

1. Under instructor's supervision, student will perform
    a. Venipuncture, using syringe technique
    b. Venipuncture, using Vacutainer technique
    c. Capillary puncture
    d. White blood cell counts
    e. Hematocrit
    f. Hemoglobin
    g. Thin blood smear preparation
    h. Wright's staining procedure
    i. Differential cell counts
    j. Thrombocyte counts
    k. Reticulocyte counts

2. Students will be responsible for maintenance of reagents and desk equipment.

3. Under instructor's supervision, student will conduct daily maintenance of microscope and mechanical devices.

4. Student will record the results of daily practical exercises on forms provided, and hand them in to be checked by instructor.

E. Safety Precautions

1. Instructors will be especially watchful for:

a. Breaks in aseptic technique (when students are performing venipuncture)

b. Students showing signs of nausea, fainting and/or illness

2. Instructors will be familiar with emergency first aid procedures in treating syncope, and with SOP in obtaining medical assistance when necessary

3. Instructors will insure that all equipment is operational, and remove any defective equipment from classroom for maintenance.

F. Questions From Students

III. SUMMARY (10 min)

A. Review of Main Points

1. Venipuncture

2. Capillary puncture

3. Complete blood count

4. Differential cell counting

5. Special hematological procedures

6. Reporting results

B. Closing Statement: Your experience gained in this classroom is an excellent beginning, but only a beginning. Many additional hours of practice will be necessary before you gain complete mastery of the techniques and procedures needed to be a competent technician in the hospital laboratory.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
CLINICAL MICROSCOPY BRANCH

*LP 08-280-034
075

Written Examination and Critique I (4 hours)

COURSES PRESENTED TO: 311-92B20 Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES:

TM 8-227-4
Miale, John B., Laboratory Medicine Hematology, 3rd ed. St. Louis: C. V. Mosby, 1967.
Wintrobe, Maxwell M. Clinical Hematology. 6th ed. Philadelphia: Lea and Febiger, 1967.

STUDY ASSIGNMENT: Before class, review all previous notes and reading assignment

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: None

PERSONNEL: One primary instructor (MOS 92B4H, 92B3H)

INSTRUCTIONAL AIDS: P.A. set with lapel microphone, chalk, chalkboard and eraser

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Written examination

I. INTRODUCTION (5 min)

A. Opening Statement: In order for a laboratory specialist to correct errors in technical procedures or make allowances for differences in the quality or quantity of reagents and/or specimens, he must understand the theory behind each test procedure. He must know the established normal range for each procedure to be able to recognize variations from normal. This is the hallmark of the competent technician.

B. Objectives

1. Answer questions on theories, principles and procedures presented in lecture and reading assignments.

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*This lesson plan supersedes LP 08-280-034, 014.

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2. Demonstrate an ability to calculate and interpret the results of hematological test procedures given specific and example test data.

C. Class Procedure and Lesson Tie-In

1. Four of 128 hours of hematology.

2. Reteach and conferences, as necessary, to solve individual problems.

II. APPLICATION (170 min)

A. Arrangement of Students

1. Students will be spaced as conditions and number of students permit during written examinations.

2. Students will be at their regularly assigned work area during reteach sessions.

B. Directions to Students

1. No written or printed material of any type will be permitted during written examinations

2. No talking allowed during written examinations

3. Check chalkboard at the beginning of each period for special instructions

4. During reteach sessions students are encouraged to ask questions

C. Instructors' Activities

1. Space students for written examinations as conditions and number of students allow

2. Issue test booklets and answer sheets (one each per student)

3. Grade answer sheets and record results immediately

4. Answer student questions during reteach sessions and reteach as indicated by examination results.

5. Circulate in classroom during examinations

6. Maintain classroom control

D. Students' Activities

1. Students will record answers on form provided by instructor and hand this form and test booklet in to instructor

2. Students will be responsible for work area

III. REVIEW (5 min)

A. Questions From Students

B. Review of Main Points Covered During Reteach Session

C. Closing Statement: You have now completed the hematology portion of the Basic Medical Laboratory Procedures course. The experience and procedures that you have learned up to this point are a good beginning, but only a beginning. It is your responsibility in the weeks and months ahead to master the material presented to become a valued member of the laboratory service.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-035
LABORATORY SCIENCES DIVISION 075
CLINICAL MICROSCOPY BRANCH

Practical Examination and Critique I (30 hours)

COURSES PRESENTED TO: 311-92B20, Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES:

TM 8-227-4.
Miale, John B. Laboratory Medicine Hematology. 3rd ed. St. Louis: C. V. Mosby, 1967.
Wintrobe, Maxwell M. Clinical Hematology. 6th ed. Philadelphia: Lea and Febiger, 1967.

STUDY ASSIGNMENT: Before class, read and review all previous notes and reading assignments.

STUDENT UNIFORM AND EQUIPMENT: Not applicable.

TOOLS, EQUIPMENT, AND MATERIALS: None.

PERSONNEL: One primary instructor (MOS 92B4H, 92B3H, or 92B2H)

INSTRUCTIONAL AIDS: P. A. set with lapel microphone, chalk, chalkboard, and eraser.

TROOP REQUIREMENTS: None.

TRANSPORTATION REQUIREMENTS: None.

METHOD OF INSTRUCTION: Practical examination.

I. INTRODUCTION (5 min)

A. Opening Statement: Any time you, as a laboratory technician, are called upon to do a complete blood count, it is your responsibility to report the final results to the best of your ability. Your capacity to perform accurate work will be greatly enhanced by applying the principles and techniques you have learned in the lectures and practical exercises to this examination.

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*This lesson plan supersedes 08-280-035, 074.

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B. Objectives

1. Demonstrate dexterity utilizing various equipment (microscope, pipets, slides, hemacytometer, lancets, mechanical, and automated devices) with a degree of accuracy equivalent to at least 70 perce

2. Assess skill in performing tests of a hematological nature (complete blood count, WBC count, hemoglobin, hematocrit, differential cell count, morphology evaluation) under laboratory conditions.

3. Evaluate proficiency reporting the results of various hematologica examinations in correct format using the proper units of report.

C. Class Procedure and Lesson Tie-in

1. Practical examination (graded) without help other than notebook or issued texts.

2. Questions must be asked before starting examination.

3. Testing will continue over a three and one-half school day period and is subdivided into CBC testing, capillary puncture, and leukocyte identification.

4. Order of presentation is dependent on specimen availability.

5. Reteach and conferences, as necessary, to solve individual problems.

II. APPLICATION (1340 min)

A. Arrangement of Students

1. Students will be at their regularly assigned work area during the practical examination.

2. Students will work in pairs when the practical examination require a capillary puncture.

B. Directions to Students

1. Pick up unknown blood specimens and/or blood films from instructor.

2. Obtain special equipment and/or reagents for exercise from instructor.

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3. Use equipment and reagents at students' desks for completion of hematological procedures.

4. Remove microscope and lamp from cabinets for completion of hematological procedures.

5. Check chalkboard in classroom at the beginning of each period for special instructions.

6. No talking or discussing results.

7. Report results of testing on forms provided by instructor.

C. Instructors' Activities

1. Prior to class

   a. Prepare reagents as needed.

   b. Prepare and key unknown specimens for students' analysis.

2. During class

   a. Issue materials, reagents, and specimens as necessary.

   b. Monitor students' performance of procedures closely, making immediate corrections.

   c. Check completed report forms and discuss problem areas with students.

   d. Maintain classroom control.

D. Students' activities

1. Under instructors' supervision, student will perform:

   a. Capillary puncture

   b. White blood cell count

   c. Hematocrit

   d. Hemoglobin

   e. Thin blood smear preparation

   f. Differential cell count

g. Morphology evaluation

2. Students will be responsible for maintenance of reagents and desk equipment

3. Under instructor's supervision, students will conduct daily maintenance of microscope and mechanical devices.

4. Students will record the results of daily practical examination on forms provided and hand them in to be checked by instructor.

III. REVIEW (5 min)

A. Questions from Students

B. Review of Main Points Covered During Reteach Session

C. Closing Statement: The physician is depending on you to alert him to any abnormalities in the cell count or cell morphology which you note as you perform a procedure. In this way, you are contributing very important information for a diagnosis.

INTRODUCTION TO HEMATOLOGY

In the hematology laboratory, the equipment utilized must be scrupulously clean and accurately calibrated. The technician must be thoroughly familiar with the use and care of equipment including microscopes, centrifuges, sterilizers, and routine glassware.

USE AND CARE OF THE MICROSCOPE

Generally, in microscopic work in hematology, particularly when dealing with wet preparations, microscope stages should not be tilted. Tilting of the stage enhances flowing of fluids and results in an uneven distribution of cells. Mount the preparation to be observed on the microscope stage and move the area of the slide to be examined to a position directly over the opening in the center of the microscope stage. Once the preparation is set on the microscope stage, clamp it into position, and then move the slide around until the desired area lies beneath the perpendicular axis of the objective.

Note that as the object is moved, a movement to the left appears in the ocular as a movement to the right in the field. Because of this, the image in the ocular is reversed in position to that of the object. Briefly stated, movement to the left will be observed as movement to the right, and movement to the right will be observed as movement in the opposite direction. Backward and forward motion are similarly reversed.

Microscopes provided in the medical laboratory are usually equipped with a mechanical stage by means of which the slide is secured into position and moved about during examination. Many types of examinations in the hematology section of the laboratory necessitate that the slide be systematically, carefully, and completely examined. In order to enable thorough examination, a mechanical stage is indispensable.

Illumination

Correct illumination of the object under study is an extremely important detail. Incorrect lighting of the object may lead to inaccurate results and conclusions as well as unnecessary eyestrain. Correct lighting depends upon the proper use of the mirror and, especially, of the iris diaphragm which controls the amount of light which passes upward through the object under observation.

The mirror is double; flat on one side and concave on the other. The mirror should always be kept free of dust particles and should not be touched with the fingers. When its position is adjusted, it should be handled by touching only the frame. The reversible mirror is provided with a double hinge so that it can be tipped at any angle to obtain the maximum amount of light through the object under study. Distracting images and reflections in the field of vision are more easily eliminated by the use of the concave side of the mirror. When using low power sources of artificial light, the concave side of the mirror gives more intense illumination and should be used at all times.

The amount of light passing through the

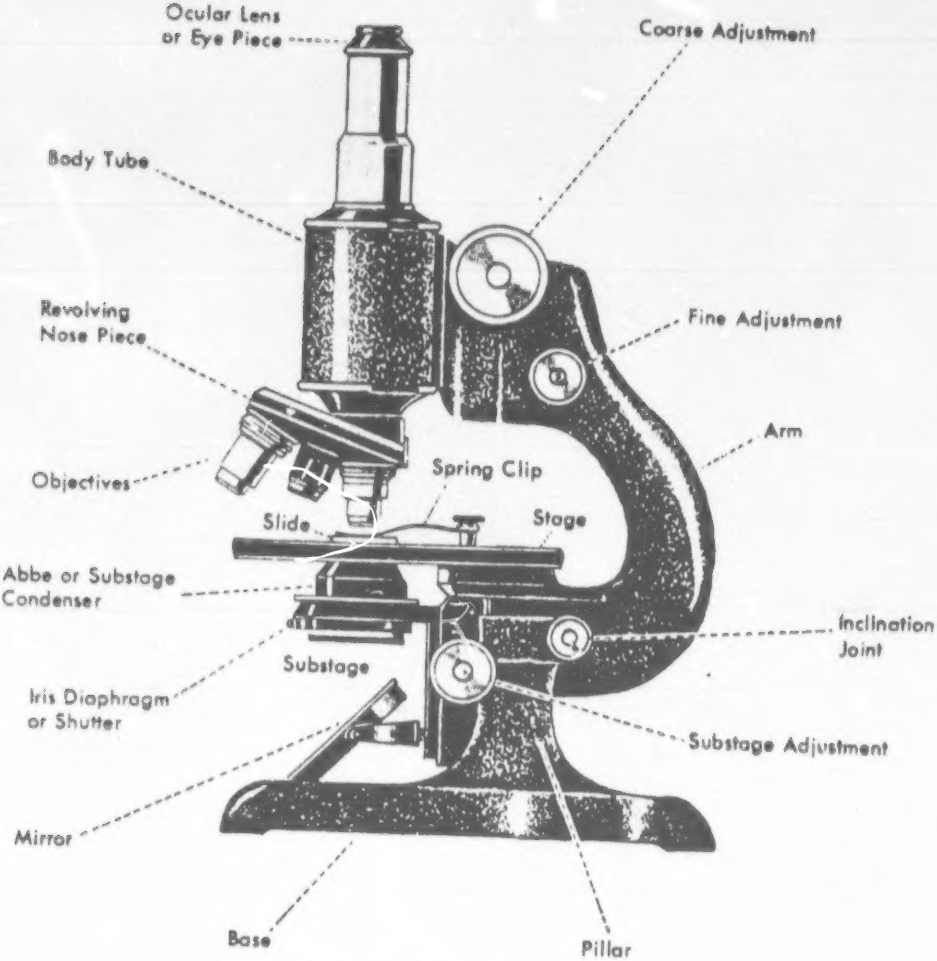
Ocular Lens or Eye Piece
Coarse Adjustment
Body Tube
Revolving Nose Piece
Fine Adjustment
Arm
Objectives
Spring Clip
Slide
Stage
Abbe or Substage Condenser
Inclination Joint
Substage
Iris Diaphragm or Shutter
Substage Adjustment
Mirror
Base
Pillar

Figure 1. A Compound Microscope.

specimen is controlled by use of the iris diaphragm in the substage condenser. The size of the opening in the diaphragm is controlled by a lever on the side of the condenser. The lever of the iris diaphragm should never be forced to the full limit in either direction. Doing so may damage the delicate leaves of the diaphragm. Generally, when observing liquid preparations under low power, the diaphragm opening should be partially closed. Under the high dry objective, the diaphragm is generally opened somewhat more to allow more light to pass through the material. When observing stained preparations under the oil immersion objective, the iris diaphragm is usually opened wide.

Various factors determine the intensity of illumination which should be used. These depend chiefly upon the individual, the intensity of the light source, whether the light source is natural or artificial, and the kind of preparation which is under observation. A general rule is that the higher the degree of magnification and the greater the density of the object under study, the greater the amount of light required to adequately illuminate the field. A major cause of failure to accurately identify blood cells is failure to maintain proper illumination. Always remember that illumination may need to be adjusted with each change of magnification and with each object which is observed for morphology.

The distance between the condenser and the stage as well as the size of the opening in the iris diaphragm need constant adjustment to accentuate details of objects observed. Too much light renders objects invisible due to glare while too little light obscures morphological details. Artificial light is better than daylight because the intensity of the light remains constant. For most purposes, artificial light should be filtered through a Corning daylight glass or cobalt glass. The size of the opening in the condenser together with its position up or down controls the light entering the system. When the condenser is close to the stage, concentration of light is greater; as the condenser is moved downward, less light passes upward through the object under observation.

Improper illumination is indicated when: (1) Dark points or shadows appear in the field; (2) the outline of an object is bright on one side and dark on the other; or (3) the object appears to be in a glare of light. These can usually be corrected by either changing the position of the mirror or reducing the amount of light by adjusting the size of the opening in the iris diaphragm.

When using the oil immersion objective, a drop of either mineral oil or oil of cedarwood is placed on the coverslip or slide. Mineral oil will not become gummy or sticky. While oil of cedarwood tends to become more gummy, it does give slightly better optical definition. To remove oil from slides and objectives, xylol is used and it is equally satisfactory for the removal of either mineral oil or cedarwood oil.

Magnification

Microscopes in general use in medical laboratories are provided with three objectives with focal lengths of 1.9 mm, 4 mm, and 16 mm, respectively. Microscopes are usually provided with 5X and 10X oculars. Multiplying the power of the ocular by the power of the objective gives the degree of magnification of the object under observation. Table I summarizes the ocular magnification, the millimeter length of the objective, its magnification power, and the total apparent increase obtained using oculars and objectives of the powers shown.

Table I. Oculars, Objectives, and Magnification Powers

Ocular	Objective	Magnification
5X	16 mm (10X)	50 diameters
10X	16 mm (10X)	100 diameters
5X	4 mm (43X)	215 diameters
10X	4 mm (43X)	430 diameters
5X	1.9 mm (97X)	485 diameters
10X	1.9 mm (97X)	970 diameters

Focusing

Focusing can be defined as the adjustment of the relationship between the optical system and the specimen so that a clear image is obtained. Several important rules to be observed when focusing the microscope on the preparation follow:

1. After the preparation to be observed is mounted on the stage, the objective to be used is turned into line with the eyepiece.
2. Movement of the objective is accomplished by revolving the nosepiece. The nosepiece is provided in order to enable rapid, convenient substitution of one objective for another. This change is effected by grasping two of the objectives between the thumb and forefinger of the right hand and rotating them until the desired objective is brought into line with the axis of the body tube. It is very important that exact alinement be obtained. The correct setting is indicated by a slight "click" as the objective comes into position.
3. Whenever the nosepiece is revolved, its movements should be observed to make certain that the objectives do not come into contact with the preparation. Some microscopes are not parfocal, that is, objects in focus under low power will not be in focus when the nosepiece

is rotated to a higher power of magnification. It may therefore, be necessary to refocus when changing to higher magnification. This prevents contact between the specimen and the lens as the objectives are rotated. In microscopes which are parfocal, it is possible to swing other objectives into place without touching the coarse adjustment and with only a slight turn of the fine adjustment knob required to restore perfect focusing.

4. To bring an object into focus, use the coarse adjustment and lower the objective until it is below the point at which the object would normally be expected to come into view. Using the coarse adjustment and at the same time looking through the ocular, raise the objective very slowly until the field comes into view. Further adjust to the best image using only the fine adjustment.
5. In focusing upward with the fine adjustment, the preparation will first appear in faint outline, then gradually more distinctly, and finally, sharply defined. If the adjustment goes beyond the point of sharp definition, it will gradually become more dim; in which case, return to the point of greatest clarity by using the fine adjustment.
6. Never move an objective downward while looking through the eyepiece. When the objective is moved downward, always observe the downward motion with the eye held level with the microscope stage (see fig. 2). Failure to observe these precautions may result in damage to the lens of the objective or the preparation under study.

Care of the Compound Microscope

The microscope is an instrument of precision with many delicate parts, and it must be handled with utmost care. The following precautions should always be observed in the care of the microscope:

1. The microscope should always be covered when not in use (see fig. 2).
2. Care should be taken to prevent all parts of the microscope from comi into contact with acid, alkali, chlor form, alcohol, or other substances whi corrode metal or dissolve the cementi substance by means of which the lens are secured into the objectives a oculars.
3. Only lens papers should be used cleaning the lenses in the oculars a objectives (fig. 2). Do not wipe lens with gauze or fibrous cloth.
4. Never touch the lenses in the ocul objective, or substage condenser wi

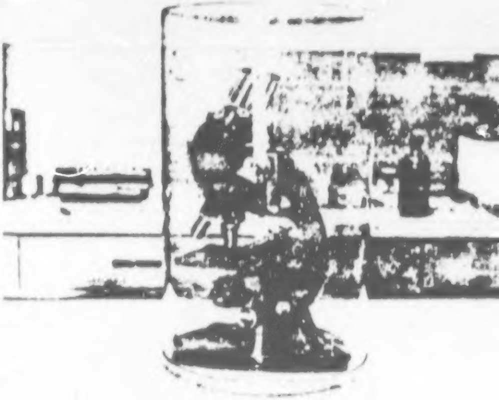

Figure 2. Care and Use of the Microscope. (1) The microsc is covered when not in use.

Figure 2.—Continued (2) Use both hands to support mircoscope

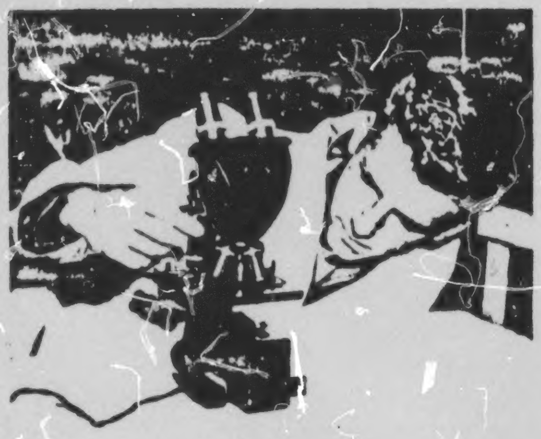

Figure 2—Continued. (3) Downward focusing is accomplished under observation.

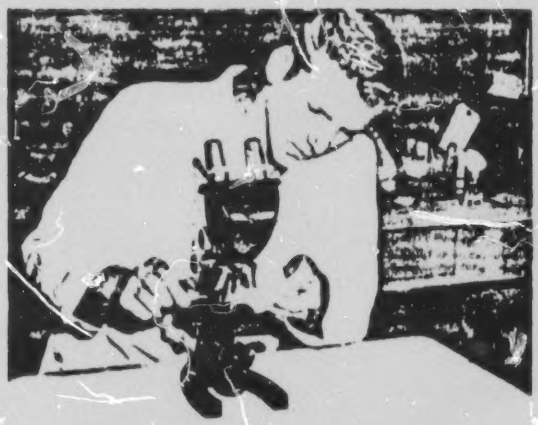

Figure 2—Continued. (4) Lenses are cleaned with xylol soaked paper.

the fingers. Even slight perspiration on otherwise clean hands may damage the lens glass which is soft and easily marred.

5. Xylol is the only agent which should be used in cleaning lenses or removing oil from objectives. Only small amounts of xylol are necessary and should be used with care.
6. When sewing machine oil is used to lubricate moving parts of the microscope, all excess should be wiped off to prevent the collection of grit and dust.
7. The microscope should be protected against direct sunlight and moisture.
8. In very warm, humid climates, microscopes should be stored in dry cabinets when not in use. Such cabinets should be reasonably airtight, equipped with a light bulb to supply heat, and several cloth bags containing a hygroscopic salt such as calcium chloride should be be placed in the chamber to absorb moisture. In warm, humid climates, the lenses of unprotected microscopes may be attacked by certain fungi which etch glass and ruin the lenses.
9. After use, always turn the nosepiece to a position which brings the low power objective into direct line with the opening in the substage condenser. If this precaution is not taken, the longer higher powered objectives may accidentally come into contact with the condenser lens.
10. In moving the microscope from one place to another, always grasp the arm of the instrument firmly with the right hand and support the base with the left hand. The proper method for carrying a microscope is illustrated in fig. 2.
11. The entire microscope should be cleaned frequently to remove dust, finger marks, oil, grease, and remnants of specimens. All parts of the microscope should be kept scrupulously clean at all times.
12. Never tamper with any of the parts of the microscope. If the instrument does not seem to be functioning properly, immediately call the matter to the attention of the laboratory officer.
13. Wide-angle lenses are now available through supply channels as are binocular microscopes. The use of these adjuncts to microscopy will greatly enhance the ease with which blood cells can be studied.
14. Maintenance of the microscope should be in accordance with the manufacturer's booklet of instruction.

CLEANING OF GLASSWARE

New glassware, such as slides, is generally covered with a thin film of grease. This should be removed by boiling slides in a 5-percent solution of sodium bicarbonate. Other glassware may be cleaned in hot, soapy water and thoroughly rinsed in distilled water. Blood dilution pipettes may be washed by flushing water and acetone through them. Ordinary household bleach may be used to remove blood clots in the bore of pipettes. Dilution pipettes are dry when the bead moves freely in the bulb. Glassware to be used for hemostasis studies must be scrupulously clean. This glassware should be soaked in dichromate-sulfuric acid solution prior to routine cleansing. The etched surface of the hemacytometer should be rinsed in water and blotted dry with lens paper to avoid marring or further etching of the lines on the surfaces.

A method of cleaning small bore tubes and pipettes (Wintrobe sedimentation rate tubes, etc.) is to attach a capillary pipette, by a rubber hose, to a water type suction pump. Attach the tube to the flat end of the pipette and hold the tube under water. Blood is drawn from the tube as water is drawn in. When the tube is clean, invert the tube, remove the residual water by suction, and allow to dry. Occasionally, the tube should be cleaned with dilute sodium hypochlorite (househouse bleach) to remove deposits of residual blood.

LABORATORY REAGENTS

Preparation of Reagents

Various stains and solutions are utilized in routine hematological examinations. These must be prepared with the utmost care and precisely according to formulations. Detailed directions for the preparations of all reagents which are required for performing procedures outlined throughout this manual are contained in the appendix. Careful attention should be given to precise measurements, order in which reagents are added, control of temperature where indicated, filtration and aging. Particular attention must be given to storage of reagents particularly with reference to requirements for refrigeration, incubation, and protection from intense light.

Labeling Reagent Containers

Proper labeling of reagents is an extremely important detail. Labels should be complete, securely attached, and neatly and legibly written or preferably typewritten. Items recorded on the label should include all constituents and quantities utilized, date of preparation, initials of the individual who prepared the reagent, and expiration date if the solution deteriorates with age. Labels should be protected against damage by water or other fluids by covering with a protective coating of warm paraffin, collodion, or shellac painted over the surface of the label.

Precautions in the Handling of Reagents

There are various precautions which must be taken in handling reagents in the hematology laboratory. Among the most important are the following:

1. Once a portion of a reagent has been removed from the original container, it should never be poured back. Doing so may contaminate the remaining unused portion.
2. Reagents are preferably stored in alphabetical order on shelving protected from dust, moisture, and direct sunlight.
3. Never use a reagent which cannot be clearly identified from the label on the container. Discard all reagents which cannot be accurately identified.

4. *Always* read the label before dispensing a reagent.
5. When working with newly prepared reagents, especially stains, ascertain whether desired results are being obtained. Unsatisfactory solutions should be discarded and replaced.
6. All mixing containers, stirring rods, and containers used for storage of reagents should be chemically cleaned prior to use.
7. During mixing and preparation, as well as in storage, it is good practice to avoid contact of reagents with metals. Many reagents contain substances which will react chemically with metal producing changes which will render them unusable for laboratory work.
8. Do not allow inexperienced personnel to prepare reagents without close supervision.
9. Certain reagents are poisonous, i.e., Drabkin's solution and bichloride of mercury, and adequate precautions should be taken to prevent accidental poisoning. All highly toxic reagents should be conspicuously labeled "POISON" and should be stored in a separate cabinet in the laboratory.

CENTRIFUGES

The centrifuge is an important item of equipment in the hematology laboratory. Several models are standard items of Army equipment. In smaller laboratories, table top instruments which accommodate six tubes at one time are usually provided. These units are mounted on rubber feet which absorb much of the vibration. The speed is controlled by means of a rheostat on the front panel. Top speeds of centrifuges will vary and the top speed of a particular instrument should be known in order to intelligently use the speed control device.

In larger laboratories, the heavier floor-mounted models are usually provided, and these can accommodate a large number of tubes at one time. The top speed of these instruments is higher than that of table models. Because of their increased inertia, they are equipped with a brake to facilitate stopping. In these units, the tubes are placed in balanced receptacles which are mounted on spokes emanating from a central hub.

A special type of high speed centrifuge is required to spin capillary tubes (fig 3). The microhematocrit centrifuge employed in hematological tests is used. After the capillary tube is filled with blood, it is closed by heat-fusing at one end (fig. 4). During centrifugation, this end is always placed in position facing toward the outside of the holder plate.

In all instances where centrifugation is required, careful attention must be given to

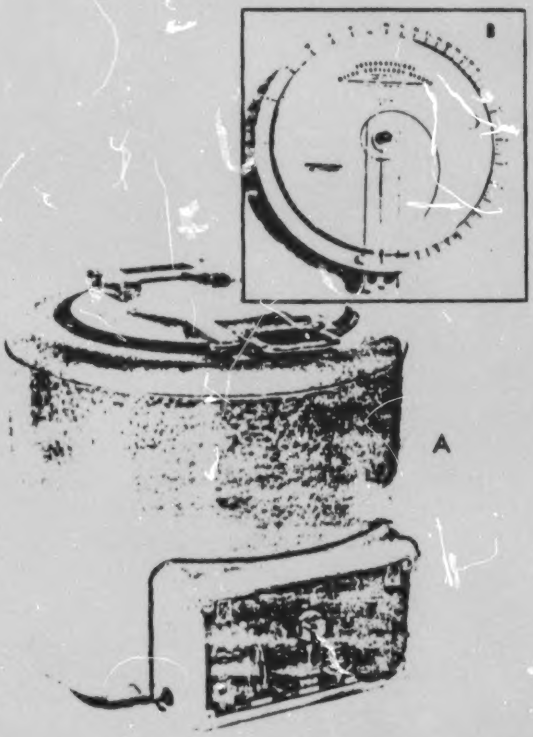

Figure 3 Microhematocrit Centrifuge (A); Microhematocrit Reader (B).

balancing the units. This means that tubes must be placed exactly opposite from each other, that they must be of identical weight, and must contain the same amount of fluid. Failure to properly balance the centrifuge probably accounts for more malfunctions than any other single factor.

If at all possible, centrifuges should be equipped with tachometers so that speed may be checked and controlled. The inside of the centrifuges should be occasionally cleaned to prevent dust particles from being blown into specimens.

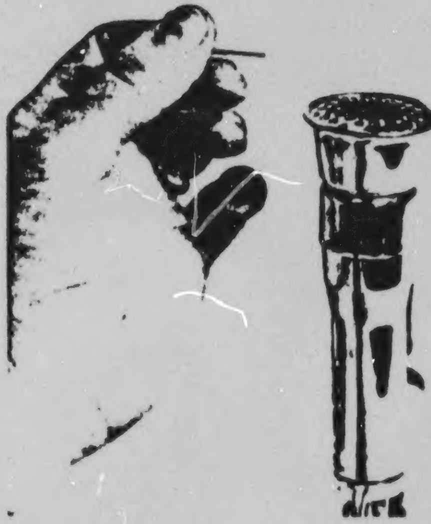

Figure 4 Sealing Capillary Tubes in Microburner Flame.

CONSTANT TEMPERATURE DEVICES

Frequently, a means of maintaining a constant temperature is required in hematological tests. If the desired temperature is above that of the room, dry air incubators or warm water baths are employed. These units are electrically operated and contain precision thermostats which hold the temperature within narrow preset limits. These devices are subject to occasional malfunction and must be carefully checked prior to use. A thermometer installed within the apparatus will reveal at a glance the operating temperature of the equipment.

Refrigeration is necessary for the routine storage of biologicals and for the performance of certain hematological procedures. Constant temperature is important and special measures must be taken to maintain this condition. Refrigerators should be opened only when absolutely necessary. For this reason, it is wise to have a separate unit for the hematology laboratory section.

STERILIZING EQUIPMENT

Certain items of equipment used in hematological procedures require sterilization. All items of glassware are preferably sterilized in autoclaves. Most of the moisture is vacuumed from the glassware by the autoclave, then hot air ovens are used to insure complete dryness. The time for autoclaving depends on the type of material or equipment being sterilized, the thickness of packs, etc., and the time of 30 minutes is minimal with 15 to 20 pounds per square inch at 250° F. Prior to autoclaving, flasks should be plugged with cotton. Pipettes and syringes may be wrapped in paper. The proper stepwise procedure for wrapping syringes for sterilization is shown in figure 5.

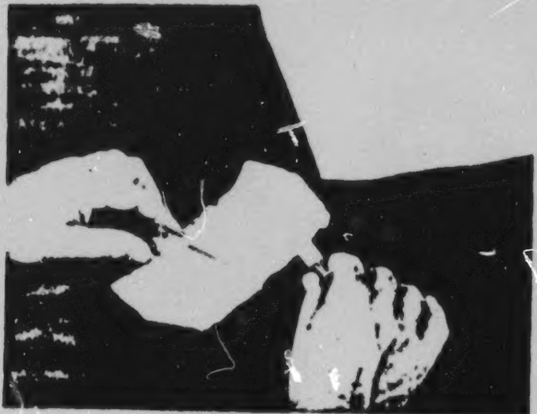

Figure 5. Procedure for Wrapping Hypodermic Syringe and Needle. STEP 1—After thoroughly cleaning and drying the syringe barrel and plunger, place the units on a 4" x 8" gauze pad.

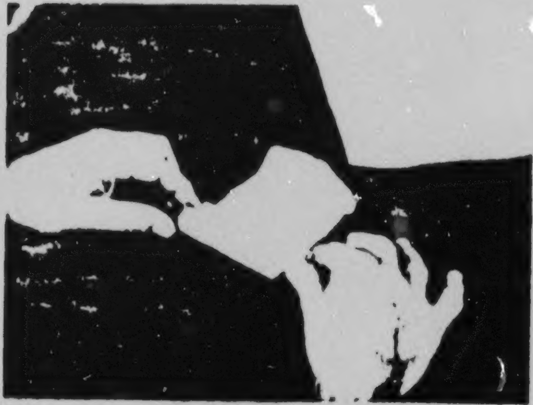

Figure 5—Continued. STEP 2—Firmly roll gauze over syringe barrel.

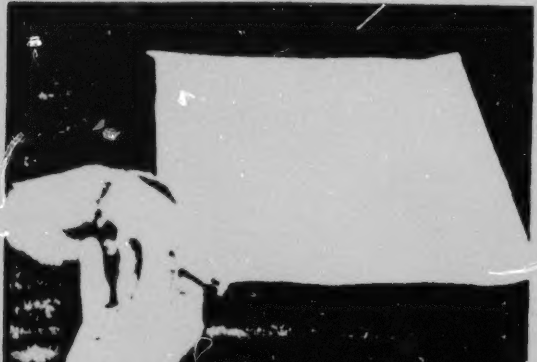

Figure 5—Continued STEP 3—Continue rolling gauze firmly over plunger.

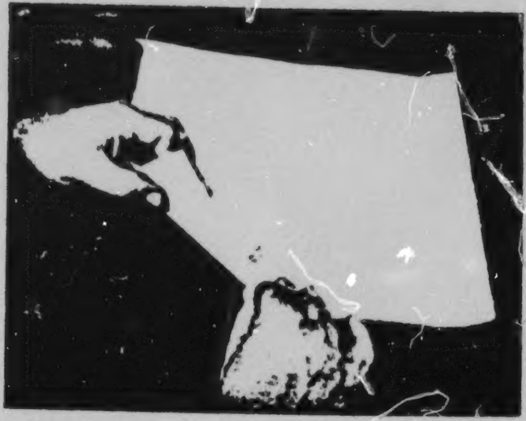

Figure 5—Continued. STEP 4—After wrapping the units in gauze, place them on the corner of a piece of heavy paper approximately 10" x 14" in size. Roll the corner of the paper and anchor it firmly beneath the equipment.

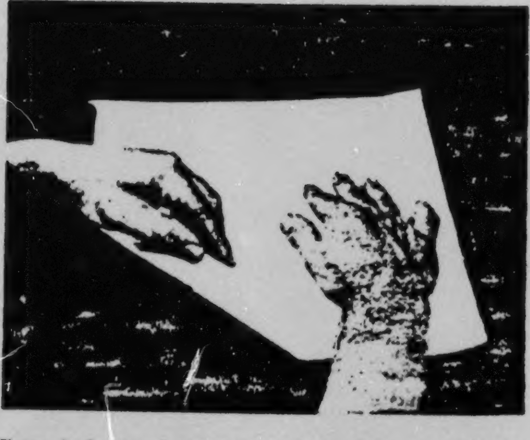

Figure 5—Continued. STEP 5—Continue rolling the equipment into the paper by rolling toward the opposite corner. When about two-thirds of the paper is rolled, fold the two other corners across in the opposite direction, then continue rolling into a firm package. Seal the package with gummed tape. Place needle in cotton-plugged glass tube.

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To sterilize equipment in a hot air oven, prepare the equipment as for autoclave sterilization, then place it in the hot air oven and gradually raise the temperature to 170°C. This is sufficient to turn white paper to a faint yellow color. Overheating should be avoided in order to prevent charring of cotton and paper and release of oil from the cotton. The cotton plugs should not be placed in contact with the walls of the oven. The material should be heated for 1½–2 hours at the above specified temperature. To prevent cracking due to too sudden contraction of the glass, allow time for the sterilizer to partially cool before opening the door.

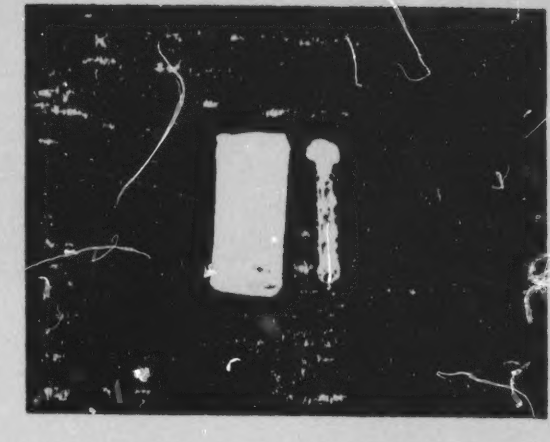


Figure 5—Continued. STEP 6—Syringe and needle properly packaged for sterilization.

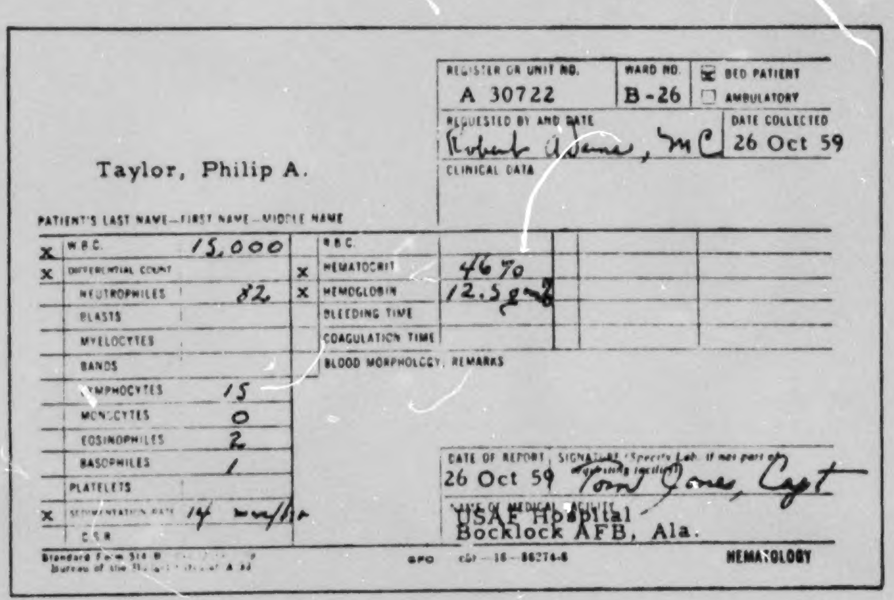
LABORATORY RECORDS AND REPORTS

Purpose

Reports of laboratory hematological examinations serve two principal purposes: they furnish useful information to the clinician for diagnosis, and they serve as a guide for treatment and followup to determine effectiveness of treatment.

Initiation of Requests

Requests for laboratory examination of specimens are initiated by the attending physician. SF 514–B, "Hematology," properly filled out and signed is submitted to the laboratory in duplicate. A sample copy of this form with the completed laboratory report is shown in figure 6. Blood cell counts



REGISTER OR UNIT NO. A 30722
WARD NO. B-26
☒ BED PATIENT
☐ AMBULATORY
REQUESTED BY AND DATE Robert A Jones, MC
DATE COLLECTED 26 Oct 59
CLINICAL DATA

Taylor, Philip A.
PATIENT'S LAST NAME—FIRST NAME—MIDDLE NAME

x	W.B.C.	15,000		R.B.C.	
x	DIFFERENTIAL COUNT		x	HEMATOCRIT	46%
	NEUTROPHILES	82	x	HEMOGLOBIN	12.5 gm%
	BLASTS			BLEEDING TIME	
	MYELOCYTES			COAGULATION TIME	
	BANDS			BLOOD MORPHOLOGY, REMARKS	
	LYMPHOCYTES	15			
	MONOCYTES	0			
	EOSINOPHILES	2			
	BASOPHILES	1			
	PLATELETS				
x	SEDIMENTATION RATE	14 mm/hr			
	C.S.R.				

DATE OF REPORT 26 Oct 59
SIGNATURE (Specify Lab. if not part of reporting facility) Tom Jones, Capt
NAME OF MEDICAL FACILITY USAF Hospital
Bocklock AFB, Ala.

Standard Form 514-B
Bureau of the Budget
GPO
HEMATOLOGY

Figure 6. Standard Form 514–B, Hematology.

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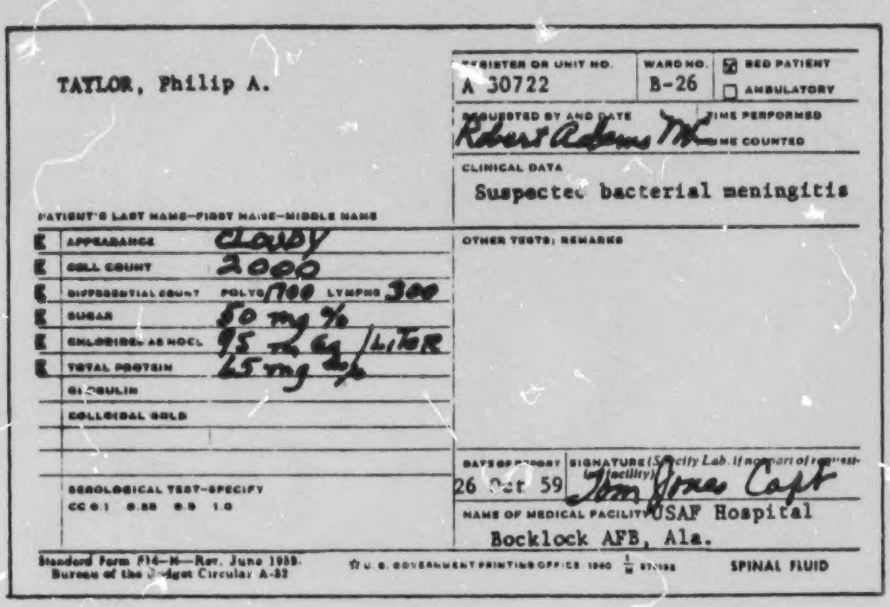

TAYLOR, Philip A.

PATIENT'S LAST NAME—FIRST NAME—MIDDLE NAME

REGISTER OR UNIT NO. A 30722

WARD NO. B-26

☒ BED PATIENT

☐ AMBULATORY

REQUESTED BY AND DATE Robert Adams MC

TIME PERFORMED

TIME COUNTED

CLINICAL DATA Suspected bacterial meningitis

OTHER TESTS; REMARKS

APPEARANCE	CLOUDY
CELL COUNT	2000
DIFFERENTIAL COUNT	POLYS 1700 LYMPHS 300
SUGAR	50 mg %
CHLORIDES AS NaCL	95 m Eq/LITER
TOTAL PROTEIN	65 mg %
GLOBULIN	
COLLOIDAL GOLD	

SEROLOGICAL TEST—SPECIFY

CC 0.1 0.25 0.5 1.0

DATE OF REPORT 26 Oct 59

SIGNATURE (Specify Lab. if not part of requesting facility) Tom Jones Capt

NAME OF MEDICAL FACILITY USAF Hospital Bocklock AFB, Ala.

Standard Form 514—H—Rev. June 1959.
Bureau of the Budget Circular A-32

☆ U. S. GOVERNMENT PRINTING OFFICE: 1960

SPINAL FLUID

Figure 7. Standard Form 514H, Spinal Fluid.

on spinal fluid are requested on SF 514h, "Spinal Fluid," which is illustrated in figure 7. When the clinician desires that special tests or certain specific observations be performed, he will usually make an entry to this effect in the remarks section on the request form, or will make an entry under the item "other tests or examinations."

Log Record

A log should be maintained in each section of the laboratory. This log is a chronological record of specimens received and results of examinations. When a specimen is brought into the laboratory, it is assigned a number which is posted in the log. Prior to submitting the laboratory report indicating findings in an examination, results are posted to the record book or cards. Log records are conveniently maintained in bound, lined ledgers with column headings written across two facing pages. Suggested column headings for entry in the daily log include date specimens submitted, specimen number (assigned by section chronologically for each day's work), name of patient, patient's status (ward number, if hospitalized; out-patient; or other), requesting physician, remarks (copy from remarks section of SF 514-B), findings and examiner's initials. The log serves as a very useful record particularly when specimens require examination procedures necessitating processing over a period of several days.

Completion of Entries and Disposition of Report Forms

Upon completion of the appropriate entries in the laboratory report slips, the original copy in cases of hospitalized personnel or out-patients is returned to the office of origin where it is filed with the patient's clinical record. Duplicate copies are retained in the laboratory office files. These serve as a permanent file record for reference purposes and reflect complete data on all examinations performed. Reports should be neat, concise, accurate, and complete. Whenever possible every effort should be made to examine a specimen and submit the report on the date the specimen is received for ex-

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amination. Entries on the laboratory reports are generally made by the technician or officer making the examination. All laboratory reports should be signed by the laboratory officer or his delegated representative. Information concerning laboratory tests should be regarded as "medical sensitive data" and confidential. Such information should be released only through proper medical channels and caution is a "must" in giving information concerning results of tests over the telephone.

SPECIMENS

Morphological studies generally should not be accomplished on blood subjected to in vitro anticoagulants as anticoagulants tend to enhance autolysis and disintegration of the cells. Delays in performing hemostasis tests should take into consideration the effect of delay on the particular test being performed. In certain tests the specimen must be processed immediately, while in others a minimal period of delay is permissible. Generally, maximum delay should not exceed 1 hour since certain coagulation factors tend to lose their potency beyond that time. Extreme care should be exercised in handling pathologic specimens for they may be infectious. The technique for obtaining and handling hematological specimens is set forth in chapter 3.

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Chapter 1

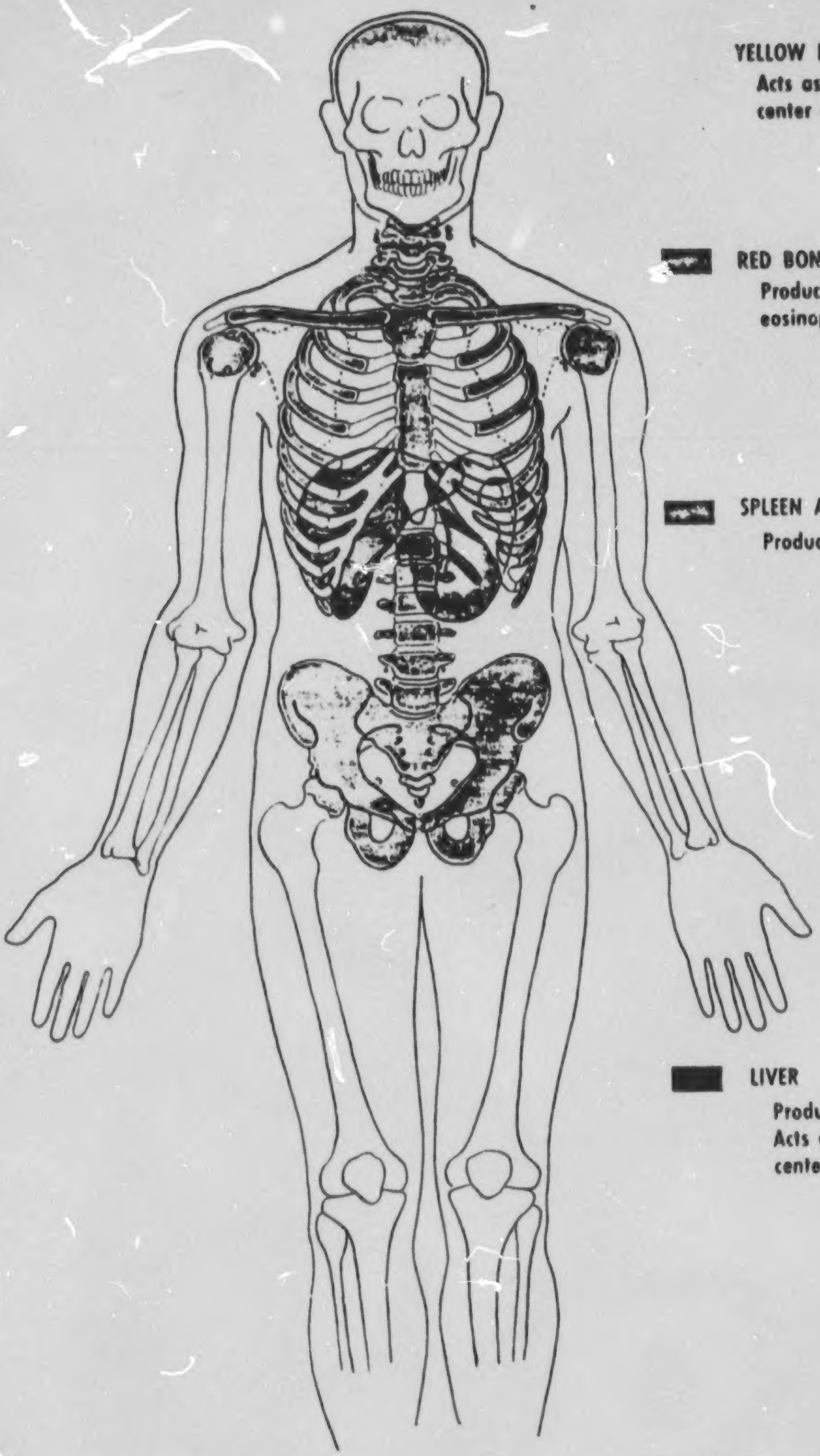
THE COMPOSITION AND FUNCTIONS OF BLOOD

Blood in its liquid state contains a fluid called plasma plus microscopically visible cellular elements. The cellular elements are leukocytes (white blood cells), erythrocytes (red blood cells), and thrombocytes (platelets). Leukocytes are true cells with well developed nuclei and possess the powers of locomotion and phagocytosis. Mature erythrocytes are nonnucleated cells which carry oxygen and carbon dioxide. The hemoglobin content of erythrocytes imparts a red color to the blood. The platelets are cellular particles associated with the clotting of blood. Plasma is a complex, slightly viscous fluid which contains proteins, carbohydrates, lipids, hormones, antibodies, enzymes, blood coagulation factors, electrolytes, and other substances.

Blood exists in vivo as a fluid medium and it performs numerous functions which are dependent upon a proper combination of all its components. As the blood circulates it comes into contact with most of the tissues and organs of the body. Malfunctions and diseases of tissues and organs often result in changes in the blood which may be diagnostic or supportive evidence of these abnormal states. Thus, the technician must be familiar with the normal blood picture in order to recognize abnormal conditions which are indicative of disease.

In the first trimester of prenatal development the blood cells are formed outside of the fetus in the yolk sac. During this period only nucleated red blood cells are produced. During the second trimester blood cell development shifts to the body of the fetus as the organs of the reticuloendothelial system (liver, spleen, thymus, etc.), are formed. During this stage red cells begin their normal development. Their development is followed by the formation of white cells and finally the thrombocytes. In the final trimester blood cells commence to be formed in the bone marrow and lymphatic system which at the time of birth constitute the total sources of hematopoiesis. The bone marrow is the principal source of production of red cells and white cells of the granulocytic series. The lymph nodes are the primary sites of production of lymphocytes, monocytes, and plasma cells.

At birth the central medullary structure of bones is red bone marrow and it is actively engaged in hematopoiesis. At about five years of age a nonhematopoietic type of marrow (fatty yellow bone marrow) which is a reserve potential tends to replace most of the red bone marrow. This partial replacement of red marrow is complete when the individual reaches maturity (about 18 years of age) at which time active hematopoietic centers in bone tissue are limited almost exclusively to the sternum, pelvic area, vertebrae, skull, ribs, clavicles, scapuli, and the epiphysis of the long bones (fig. 1).



YELLOW BONE MARROW
Acts as extramedullary hematopoietic center on demand.

▨ **RED BONE MARROW**
Produces neutrophils, basophils, eosinophils, platelets and erythrocytes.

▨ **SPLEEN AND LYMPH NODES**
Produce monocytes and lymphocytes.

■ **LIVER**
Produces prothrombin and fibrinogen. Acts as extramedullary hematopoietic center on demand.

Figure 1. Schematic Diagram of the Hematopoietic System in an Adult 18–20 Years of Age.

BLOOD CELLS

There are wide differences of opinion concerning the exact origin of blood cells. Ultimately, all blood cells develop from the undifferentiated mesenchyme of the reticuloendothelial system.

In the study of the maturation sequence of blood cells, consideration should be given to cell size; nuclear characteristics, including size, shape, color, and chromatin structure; and cytoplasmic characteristics, including quantity, color, and granules.

Since all blood cells originate from stem cells or undifferentiated mesenchymal cells, the most primitive cells are morphologically similar. Generally, they cannot be distinguished one from another on the basis of appearance alone. Their nomenclature is based on the tissue in which they are formed, the cells with which they are associated, and the cells which they are destined to produce. The same basic changes, from primitive to mature cells, occur in most types. Young cells, generally, are large and tend to become smaller as they mature. The cytoplasm tends to stain a dark blue in the immature stages of development and to stain a lighter color (blue, gray, or buff) as the cells mature.

As the cells mature the nucleus gradually becomes smaller, stains darker, and the chromatin meshwork becomes "coarse" with the strands of chromatin less fine and lacelike. In the course of cell development the nucleus changes its shape, particularly in the granulocytic series, where it becomes indented, lobulated, segmented, or fragmented. As deterioration progresses the nucleus, if still intact, becomes small, compact, usually dark, and structureless and the nucleus may completely disappear (see table I).

Cytoplasm of Blood Cells

The cytoplasm in the immature cell stains dark blue (basophilic) but tends to become lighter (or more acidophilic) as the cell develops. Granules are not present in the earliest cells and specific granules do not develop in the erythocytic, lymphocytic, monocytic, and plasmocytic series. Azurophilic and nonspecific granules are occasionally found in lymphocytes and monocytes.

Blood Cell Nuclei

The nucleus of the young cell is large, round, and occupies most of the cell. As the cell matures, the size of the nucleus decreases. Nuclei of early or primitive cells usually have one or more nucleoli. The latter are small, round, homogeneous areas which usually stain light blue with a darker boundary. In appearance nucleoli are somewhat like craters in the nucleus. They are surrounded by strands of chromatin. These nucleoli, plus a delicate reticular network of chromatin, are the principal indicators of blood cell immaturity.

Blood Cell Maturation

Mitotic figures and irregular marginal cytoplasmic tags are signs of immaturity. These irregularities may be due to the tearing away of the fixed and nonmotile primitive cells from their attachments or to the violence of cell division. Lobulation and segmentation of nuclei and blunt cytoplasmic pseudopods are also evidence of cell maturity. Phagocytosis of particulate matter is characteristic of mature differentiated cells.

Table I. Development of Blood Cells.

	LEUKOCYTES						ERYTHROCYTES	THROMBOCYTES
	Granulocytic Series			Lymphocytic Series	Monocytic Series	Plasmocytic Series		
BLOOD FORMING ORGANS	Myeloblast			Lymphoblast	Monoblast	Plasmoblast	Rubriblast	Megakaryoblast
	Progranulocyte						Prorubricyte	Promegakaryocyte
	Neutrophilic Myelocyte	Eosinophilic Myelocyte	Basophilic Myelocyte				Rubricyte	Megakaryocyte
				Prolymphocyte	Promonocyte	Proplasmocyte		
	Neutrophilic Metamyelocyte	Eosinophilic Metamyelocyte	Basophilic Metamyelocyte				Metarubricyte	Metamegakaryocyte
						Plasmocyte	Reticulocyte	
PERIPHERAL BLOOD	Neutrophilic Band	Eosinophilic Band	Basophilic Band					
	Segmented Neutrophil	Segmented Eosinophil	Segmented Basophil	Lymphocyte	Monocyte		Erythrocyte	Thrombocyte

BLOOD PLASMA

Blood plasma is the fluid portion of the blood before clotting has occurred. The phenomenon of coagulation is primarily a plasmatic process although it is modified by the presence of platelets and other substances. Serum is the fluid portion of blood remaining after the coagulation process is complete (removal of fibrinogen).

Plasma is obtained from blood by mixing an anticoagulant with the blood, then centrifuging or allowing the cells to settle leaving the clear supernatant plasma. In the laboratory plasma is often referred to by a prefix indicating the anticoagulant used, i.e., oxalate-plasma, heparin-plasma, etc.

Blood is approximately 60 percent plasma by volume. The chemical nature of plasma is very complex. Plasma is composed mostly of water but it also contains dissolved gases (oxygen, carbon dioxide, and nitrogen), proteins (albumin, globulin, and fibrinogen), carbohydrates (glucose), lipids (fats, lecithin, and cholesterol), nonprotein nitrogenous substances, and inorganic salts. The above substances are found in varying quantities under differing conditions.

VARIATIONS IN CELL COUNTS

As an individual becomes older metabolic needs change and this alteration of requirements is accompanied by a change in numbers of the different types of blood cells. The changes which normally occur are diagrammatically presented in figures 2 and 3.

FUNCTIONS OF BLOOD

The functions of blood are intimately associated with the processes of life. As such, any discussion of this very broad subject is necessarily extremely complex. For purposes of this manual blood is briefly discussed in regard to two major functions; namely, metabolism and protection.

Blood supplies the cells of the body with nutrient material to maintain cell life and function. Nutrient materials include food, water, salts, oxygen, etc. Whole blood also transports waste products such as carbon dioxide and lactic acid from the cells to the excretory organs.

Antibodies against causative agents of disease are produced by the hematopoietic system and distributed throughout the body in the blood. Leukocytes are capable of engulfing foreign substances such as bacteria and viruses. Also, the blood contains substances which promote the clotting process when injury causes bleeding or hemorrhage.

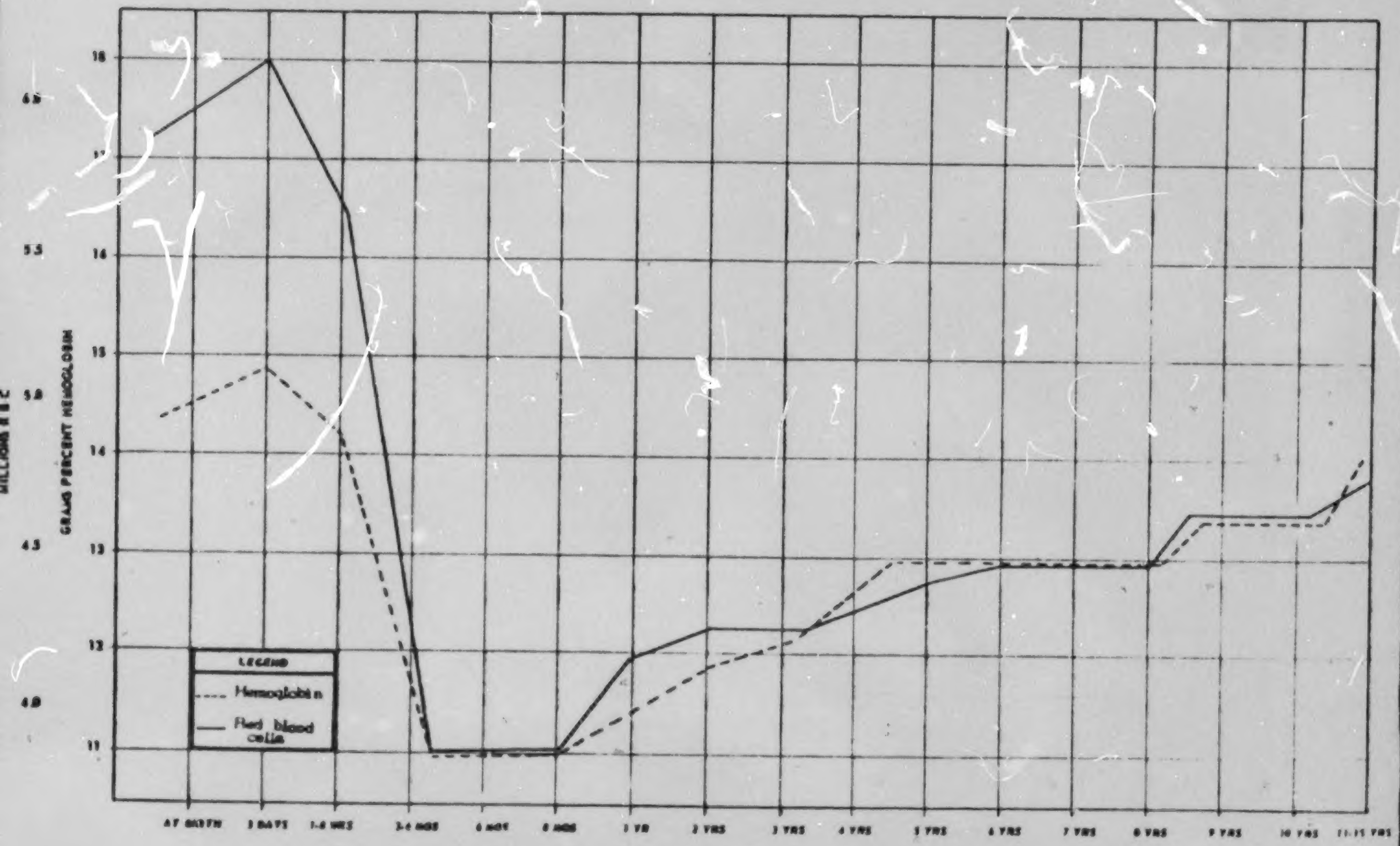


Figure 2. Variations in Hemoglobin and Red Blood Cells by Age Groups.

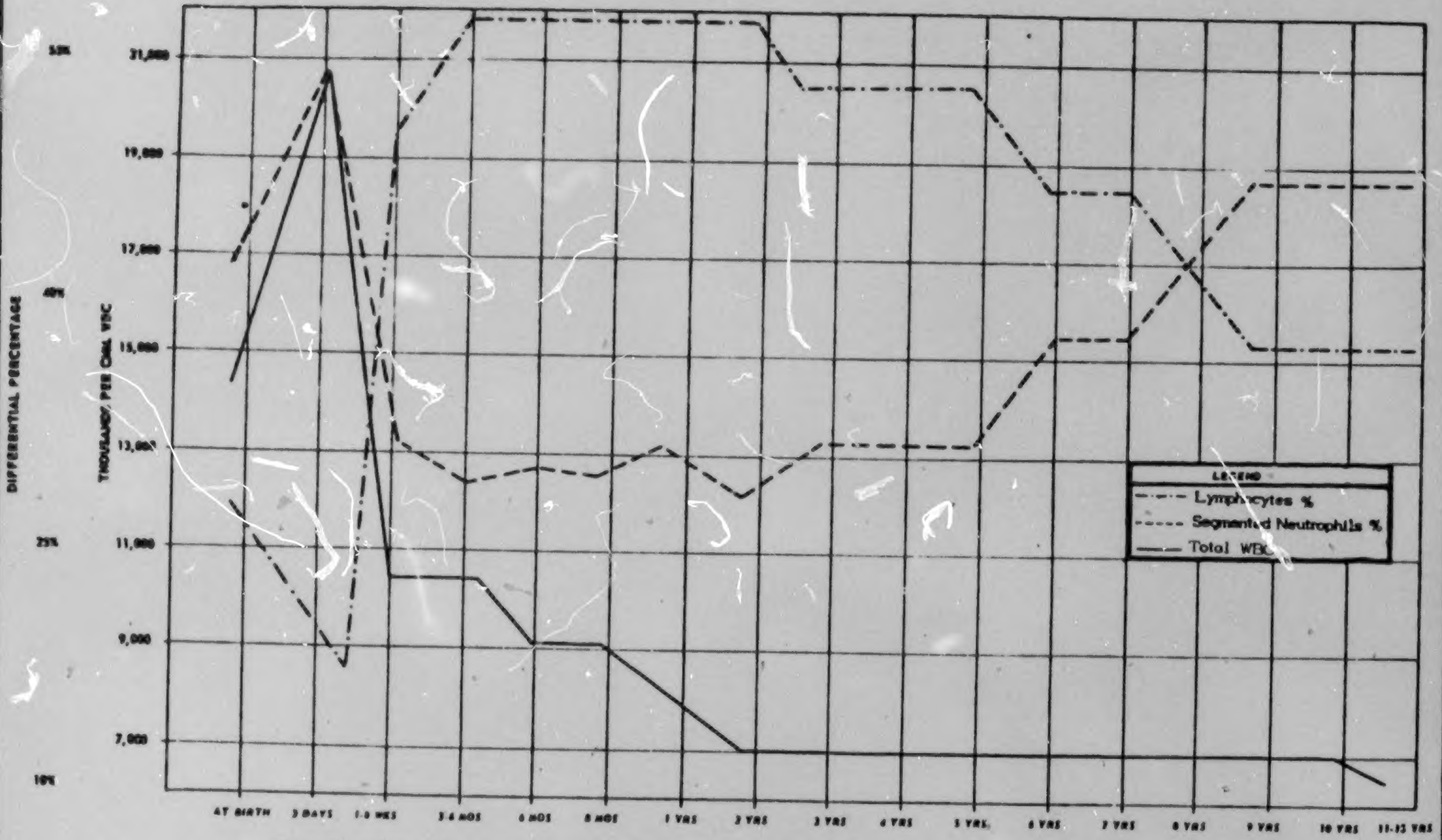


Figure 3. Relationship Between Age, and Differential Percentage and Absolute Counts of White Blood Cells.

Chapter 2

BLOOD CELL COUNTING CHAMBERS AND DILUTION PIPETTES

Blood is composed of red cells, white cells, and thrombocytes suspended in a fluid medium called the plasma. The number of cells of the various types, per given volume of blood can be determined by counting the cells in a measured quantity of diluted blood. The comparison of normal cell counts with those found in abnormal conditions is of value to the clinician in the evaluation of disease processes. Because of the large numbers of cells present in a cubic millimeter of blood, it is necessary to dilute the blood specimen. Dilution is accomplished by the blood cell diluting pipette. The hemacytometer counting chamber gives the volume.

BLOOD CELL COUNTING CHAMBERS

Numerous types of counting chambers with various types of markings have been designed. The most widely used type is the Spencer "Bright-Line" hemacytometer with thin, semitransparent lines on the glass surface. Lines ruled through the glass stand out as bright lines against a gray background with a minimum of glare, hence the designation, Bright-Line Hemacytometer.

Coverglasses made especially for cell counting should be used. Ordinary coverglasses such as those used for blood smears are unsatisfactory because they may have defects and are not heavy enough to insure correct depth of the blood specimen in the counting chamber.

As noted in figure 1, chapter 5, each large square in the ruled area equals 1 sq mm, thus there are 9 sq mm represented on the drawing. In the technique of cell counting, calculations are estimated on the average of 1 sq mm, thus if 4 large areas are counted, the average would be obtained by dividing by 4; if 9 large areas are counted, then divide by 9.

The depth of the chamber is 1/10 mm, therefore it is necessary to multiply by 10 to arrive at a corrected depth of 1 mm. This applies only to the hemacytometers which have a depth of 1/10 mm. Some hemacytometers are calibrated to a depth of 2/10 mm, and when such chambers are used, instea of multiplying by 10 to correct for depth, one must multiply by 5. Thus, the mathematical correction factors for volume in the hemacytometer are the area factor and th depth factor.

$$\text{Volume} = \text{Length} \times \text{Width} \times \text{Depth}$$

$$\text{therefore, Volume} = 1 \times 1 \times 1 = 1 \text{ cubic millimete}$$

All counting chambers should be clea and dry before use and should be cleanse immediately after use by washing in ta water. Alcohol followed by ether or aceton sodium bicarbonate, or soap and warm wate may be used to remove grease or dried fluid

The chamber should be dried with a clean, lint-free, soft cloth and lens paper.

Avoid harsh rubbing or strong acid or alkali cleaning solutions as they tend to mar or etch the ruled area. Gauze or linen especially should not be used for drying as these materials will scratch the glass. When cleansing the surface of the counting chamber and its cover, hold the sides so natural oils from the fingers do not soil the glass surfaces. Fingerprints on surfaces are difficult to remove and may lead to counting errors.

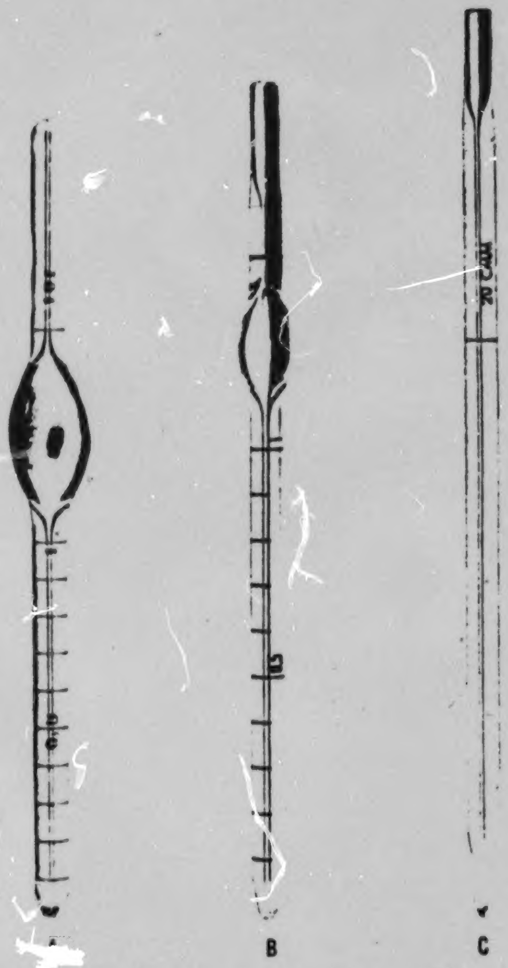


Figure 1. Hematological Pipettes. A., Red cell diluting pipette; b., White cell diluting pipette; c., Sahli pipette.

DILUTING PIPETTES

Pipettes are the instruments for performing the dilution. To dilute blood and other fluids, the Thoma type of glass pipette is most widely used. This pipette has a graduated capillary tube having a volume of one unit with calibration markings at 0.1 units, and a mixing bulb above this capillary tube. The mixing bulb contains a glass bead. Above the mixing bulb is another shorter capillary tube with an engraved mark. The pipette for performing the white blood cell count has a white bead, the mixing bulb is smaller than that of the red count pipette, and the marking above the bulb reads "11." The pipette for performing the red blood cell count has a red bead in the mixing bulb and the marking above the bulb is "101."

These pipettes are used to take the specimen directly from a skin puncture or, after careful mixing, from a vial of fluid or of blood treated with an anticoagulant such as oxalate or heparin. The blood or fluid is drawn into the pipette and diluted to the correct mark with diluting fluid. After proper mixing, the diluted substance is placed in the counting chamber and the cells are counted.

Usually, the technique for diluting the blood specimen with a pipette calls for whole blood to be drawn exactly to the 0.5 mark and diluted only to the "11" or "101" mark with appropriate diluting fluid dependent upon the type of cell count. Since the volume of fluid in the stem does not enter into the dilution, the dilution is calculated on the volume in the bulb, thus with the white blood cell count:

$$\text{Dilution} = \frac{\text{Whole blood}}{\text{Volume in bulb}} = \frac{0.5}{10} = \frac{1}{20}$$

and in the RBC count

$$\text{Dilution} = \frac{\text{Whole blood}}{\text{Volume in bulb}} = \frac{0.5}{100} = \frac{1}{200}$$

These figures represent dilution and are used as dilution correction factors by multiplication of the dilution to bring it to full concentration, i.e., the reading with 1/20 dilution must be multiplied by 20 to bring it to full concentration and the 1/200 dilution multiplied by 200.

Thoma diluting pipettes are delicate pieces of equipment and should have as careful treatment as the counting chamber. It is also important that clean, dry diluting pipettes be used to prevent dilution errors and hemolysis of cells.

If an aspirator is available, pipettes may be easily washed by drawing cold tap water (distilled water is preferred) through the pipette. If a deposit remains, it may be dissolved by drawing dilute sodium hypochlorite (household bleach) through the pipette followed by several washings with distilled water. If pipettes become plugged through neglect, the hole may be opened with a fine steel wire, and the above procedures may then be carried out. When necessary, pipettes may be dried rapidly by drawing a small amount of alcohol through them and then ether or acetone, followed by a stream of air until the glass bead moves freely and no moisture remains in the pipette.

SAHLI PIPETTES

Sahli hemoglobin pipettes are volumetric pipettes. The volume is "supposed" to be 20 cmm as registered on the side of the pipette. Normally, because this volume is not an accurate measurement, standardization of the pipette is necessary. This is usually done with mercury and the procedure is necessary to ascertain the correction factor for each pipette.

Calibration of Sahli Pipettes

APPARATUS

Analytical balance
Tuberculin or 2 ml syringe
One-hole rubber stopper, size 0 (obtain commercially)
Beaker, 50 ml
Retort stand and clamp
Thermometer
Weighing bottle

REAGENTS

Mercury (20)
Stopcock grease

TECHNIQUE

1. Heavily lubricate the barrel of the syringe with stopcock grease.
2. Tightly fit the tip of the syringe into the broad end of the rubber stopper.
3. Clamp the stopper to a heavy retort stand. Insert the base of the pipette into the other end of the rubber stopper until it is in contact with the tip of the syringe. Adjust the assembly so that the pipette occupies a vertical position.
4. Allow the mercury and the pipettes to reach room temperature before starting the calibration.
5. Determine the temperature of the mercury.

6. Slightly withdraw the plunger of the syringe.
7. Hold the beaker containing mercury so that the tip of the pipette is immersed.
8. Withdraw the plunger of the springe until mercury reaches the 20 cu mm mark.
9. Hold the barrel of the syringe steady and quickly pull the beaker of mercury away from the tip of the pipette. (Slight fluctuation in the column of mercury at this point has no appreciable significance provided there is no loss of mercury from the tip of the pipette.)
10. Expel the mercury from the pipette into a weighing bottle by manipulation of the springe. (The weight of the weighing bottle is precisely determined before starting the calibration.)
11. Prepare to weigh the mercury by sitting directly before the center of the balance.
12. Make sure the balance is level.
13. Release and arrest the beam with a slow, steady movement of the hand.
14. Arrest the beam before placing anything on, or removing anything from, the pans.
15. Find the zero point.
16. Place the weighing bottle containing mercury on the left pan.
17. Close the balance case before starting to add weights.
18. When selecting weight, the first weight is a guess; after that, if by adding the next weight the indicator swings to the right, the weight is too light, but if to the left, too heavy.
19. If they are available, add weights first by using the dials in the lower right-hand corner of the balance. These will not turn unless the beam is raised.
20. Adjust the chainometic control until the zero point is reached.
21. The weights are then added to constitute the final weight of the bottle of mercury.

CALCULATIONS

1. Determine the weight of the pipetted mercury by subtraction: (weight of weighing bottle + mercury) — (weight of weighting bottle). As an example consider the weight of the mercury to be 0.268 grams.
2. Convert grams to milligrams:

$$0.268 \text{ gm hg.} \times \frac{1000 \text{ mg}}{1 \text{ gm}} = 268 \text{ mg Hg.}$$

3. Select the temperature correction factor from table 1; i.e., the weight of one microliter of mercury in milligrams.
4. The volume of the pipette is obtained by calculating the volume of mercury.

Example:

$$\text{Wt. of mercury in mgm} \times \frac{1 \text{ microliter Hg.}}{\text{No. mg at T}^{\circ}} = \text{vol. Hg.}$$

in microliters.

$$(\text{Wt. of delivered Hg.})\ 268 \text{ mg Hg.} \times \frac{1 \text{ ul Hg.}}{13.55 \text{ mg Hg.}} = 19.8 \text{ ul vol.}$$

The expected weight for 20 cubic microliters of mercury is 270.8 mg.

Note: For routine purposes, it is acceptable to use pipettes within ± 1 percent of 20 microliters without making a correction. Therefore, pipettes which deliver 268.1 to 273.5 mg of hemoglobin would be acceptable without further calibration.

5. Should the pipette volume exceed the ± percent permissable deviation from 20 ul, a numerical factor can be derived dividing 20 by the actual volume. All hemoglobin readings, then, would be multiplied by the factor in order to obtain an accurate hemoglobin value.

Example:

Actual volume of pipette = 19.6 ul

$$\text{Numerical factor} = \frac{20}{19.6} = 1.024$$

Hemoglobin determination = 14.0 gm%

True hemoglobin value =
14.0 × 1.024 = 14.336 = 14.3 gms.

The terms microliter (ul), lambda (λ), and cubic millimeter (mm^3) are interchangeable. Microliter is becoming the more acceptable term.

1 ml = cm^3
1 ml = 1000 ul
1 cm^3 = 1000 mm^3
1000 ul = 1000 mm^3
1 ul = 1 mm^3

Table I. Apparent Weights of Mercury and Water in Air

Temperature (degrees Centigrade)	Mercury (weight of 1 ul)* mgm
20.0	13.55
21.0	13.55
22.0	13.54
23.0	13.54
24.0	13.54
25.0	13.54
26.0	13.53
27.0	13.53
28.0	13.53

*These values do not indicate the true densities and weights of water and mercury but include corrections for various factors, such as the coefficient of expansion of glass and the buoyant effect of air on the fluid weighed.

Chapter 3

COLLECTION AND PROCESSING OF BLOOD SPECIMENS

Hematological laboratory procedures are based upon the examination of blood specimens. In order to obtain valid test results, specimens must be properly collected, processed, and recorded. Blood specimens are usually obtained by either venous or capillary puncture. The source from which the specimen is taken is determined chiefly by the quantity of blood required to perform the laboratory procedures requested and the age and condition of the patient.

If a free-flowing capillary blood specimen is obtained, there is generally little difference in blood counts performed on venous or capillary blood. When capillary specimens are not taken from a free-flowing sample, or when they are obtained from areas of local stasis, cyanotic or calloused areas, valid blood counts cannot be made. White blood cell counts made on blood obtained from such sources may vary as much as 1000–1500/cmm from normal. Since there is no essential difference between venous and capillary blood, either can be used for quantitative or qualitative analyses. For general purposes, however, venous samples are preferable since they allow for multiple and/or repeated hematological examinations and provide a sufficient quantity of blood for performing any required laboratory procedure. In situations where there are limitations on the quantity of blood which can be obtained, i.e., in small infants or extensive burn cases, newly developed microquantitative methods may be entirely satisfactory for performing an analysis on a specimen obtained by capillary puncture.

OBTAINING BLOOD SPECIMENS BY VENIPUNCTURE

To obtain blood by venipuncture (see fig. 1) the specimen is drawn directly from a patient's vein with a sterile hypodermic needle and syringe. In adults the vein utilized is usually the antecubital vein of the forearm and in infants the jugular or femoral vein is punctured. The vein selected should be large, readily accessible, and sufficiently close to the surface to be seen and palpated. If venipuncture poses a problem due to the age of the patient, sclerotization due to repeated venipuncture, or any other unusual circumstance, the technician should consult the physician concerning the procedure. Under no circumstances should a technician withdraw blood where a sagittal sinus, jugular vein, or femoral vein puncture is required. This should be left to the discretion of the physician in charge.

When venipuncture is used to obtain blood for quantitative analysis, the blood must be obtained rapidly from an area which has good circulation. If the tourniquet is kept in place for longer than 2 minutes, changes

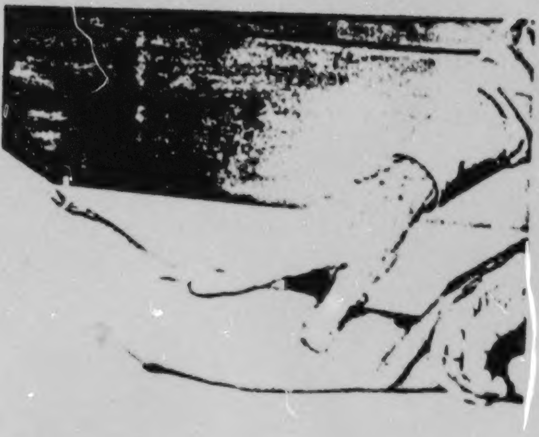


Figure 1. Obtaining Blood Specimen by Venipuncture. STEP 1—Apply a tourniquet and palpate the antecubital area for a suitable vein.

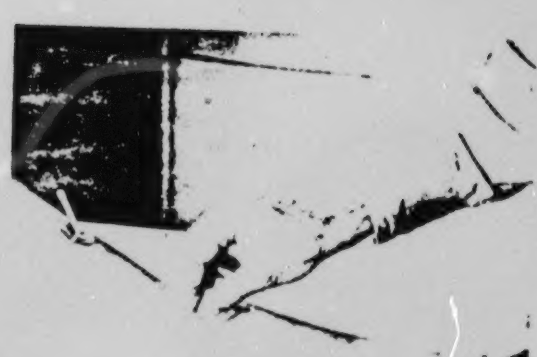


Figure 1—Continued. STEP 2—After locating a prominent and firmly anchored vein, clean the area with an alcohol sponge. A circular motion should be used.

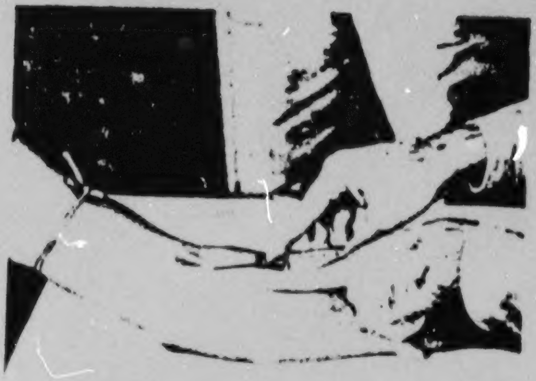


Figure 1—Continued. STEP 3—Hold the skin taut with the left hand. Grasp the syringe with the right hand and place the needle point in position at the site to be penetrated. Placing the forefinger on the needle hub aids in controlling the needle insertion.

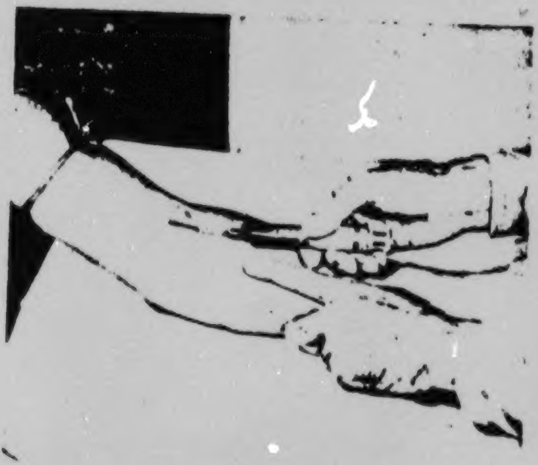


Figure 1—Continued. STEP 4—Penetrate skin and move the needle forward, parallel to and alongside the vein. A slight movement toward the vein should place it in the lumen. After entry into the vein blood will appear in the syringe.

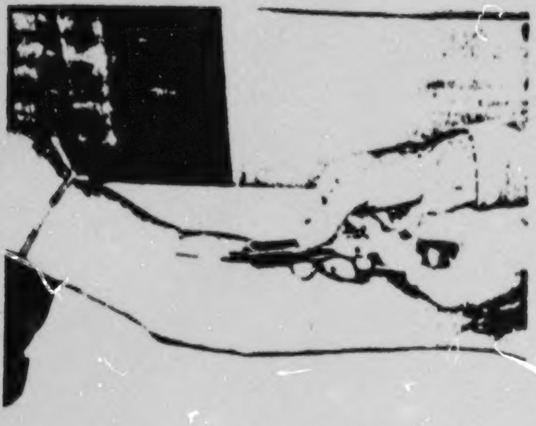


Figure 1—Continued. STEP 5—Aspiration of the blood is accomplished by gently pulling upon the syringe plunger. The syringe barrel should be held steady during this process. Withdraw the desired quantity of blood.

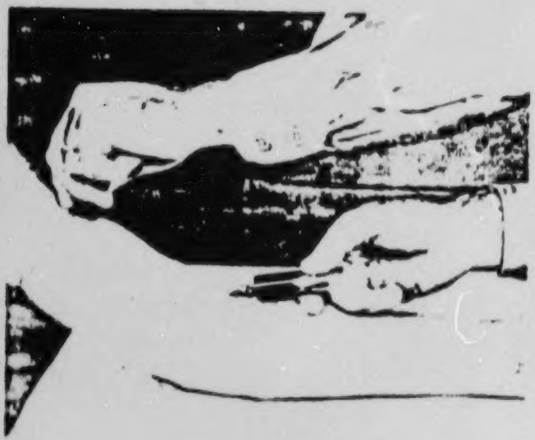


Figure 1—Continued. STEP 6—Remove the tourniquet. This must be done prior to withdrawing the needle from the vein.

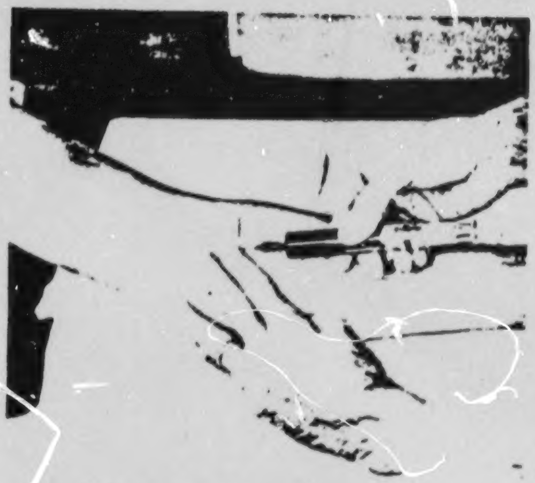


Figure 1—Continued. STEP 7—Place a sterile gauze pad over the point where the needle enters the skin and deftly withdraw the needle.

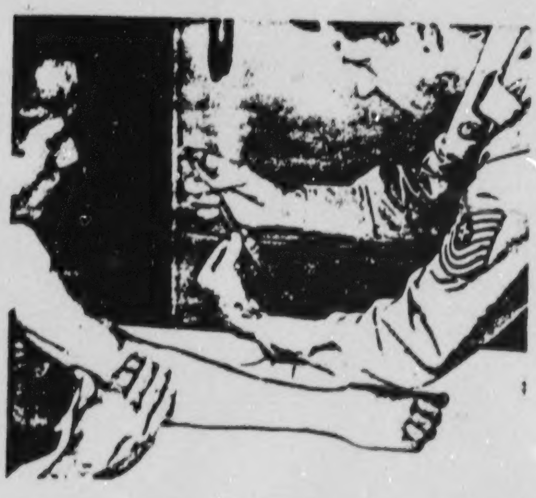


Figure 1—Continued. STEP 8—Have the patient extend the arm and maintain light pressure on the gauze pad over venipuncture site. Remove the needle from syringe and apply gentle pressure to the plunger and discharge the blood against tube margin.

may result in blood concentration. The blood should be aspirated into a clean, dry syringe without delay while the blood is still turbulent. If blood smears are required for any test required, blood can be expelled gently onto the microscopic slide from the needle. The smear should be made as soon as possible after the drop of blood is placed on the glass slide, otherwise distribution of cells will be affected by cellular migration or the sample may clot. Qualitative blood examinations, such as blood smears, should not be made from blood containing an anticoagulant. Adequate numbers of blood smears should be taken initially so that additional stained slides may be readily made without collecting more specimens. Generally two is the minimum number of blood smears to be made on any one patient.

All syringes, needles, lancets, or other instruments used for the collection of blood specimens must be sterile. Sterilization by autoclaving is preferable and recommended. This is necessary to prevent the possible transmission of homologous serum jaundice. Chemical sterilization is completely inadequate and should not be utilized.

It is most important that correct technique be practiced in order to avoid unnecessary pain to the patient, prevent tissue damage, secure a good representative blood specimen, and prevent contamination of the specimen or infection of the patient.

APPARATUS

Tourniquet
Gauze squares, 2 x 2 in.
Needle, 1½-2 in., 19-21 gauge (sterile)
Syringe with matching plunger (sterile)
Tube, collecting (with or without anticoagulant, depending upon the analyses to be performed)

REAGENTS

Alcohol, ethyl, 70 percent, or other suitable disinfectant (2)

TECHNIQUE

1. Cleanse hands thoroughly with soap and water.
2. Place an identifying label on the blood collecting tube.
3. Remove sterile syringe from wrapper and sterile needle from vial and assemble leaving the vial covering over the needle when not in use. Attach the needle so that the bevel faces in the same direction as the graduations on the syringe.
4. Holding the syringe and needle at eye level against a light background, inspect the needle bevel and point. The bevel should be smooth and shiny and the point sharply tapered. Replace damaged needles.
5. Check to be sure that the syringe works smoothly and that the needle is not plugged or burred. The syringe and needle must be dry to avoid hemolysis of the red cells. The plunger must match the syringe and must be pushed firmly to the bottom of the cylinder to prevent injection of air into the vein.
6. Place a tourniquet around the patient's arm above the elbow tightly enough to check venous circulation, but not so tightly as to stop arterial flow. *Caution:* Do not allow the tourniquet to remain in place for more than 2 minutes. Check the pulse at the wrist to make sure that arterial circulation is not cut off (see fig. 1).
7. Instruct the patient to open and close the fist several times to increase circulation.
8. By inspection and palpation locate the desired vein, determine the direction of its course, and estimate its size and depth.
9. Cleanse the skin over the selected vein with gauze soaked in 70 percent alcohol. Wipe off excess alcohol with a sterile dry gauze. Do not contaminate the area after cleaning.
10. Have the patient "make a fist" and straighten the arm. Frequently it is helpful to have the patient place the clenched fist of the other hand under the elbow to straighten the arm.
11. Grasp the syringe in the right hand and place forefinger on the hub of the

needle to guide it. Graduations on the syringe should be in full view of the technician.

12. Grasp the forearm with the left hand about 2 inches below the area to be punctured and hold the skin taut with the thumb.
13. Holding the needle at approximately a 30° angle to the patient's arm, insert the needle through the skin immediately adjacent to the vein to be punctured. Lower the syringe and move the needle parallel to the vein for a distance of about one-half inch.
14. Slowly guide the needle toward the blood vessel and direct it into the lumen. A slight "give" indicates the vein has been punctured.
15. In vigorous individuals, blood will be forced back into the syringe spontaneously. If pressure in the vein is low, the plunger may have to be withdrawn slightly to make certain that the needle has entered the vein.
16. Move the needle a short distance farther into the vein to prevent it from coming out of the vessel while blood is being withdrawn. Care must be taken not to penetrate through the other side of the vein.
17. Withdraw the blood by using the left hand to pull the plunger while steadying the syringe and needle with the right hand.
18. Release the tourniquet. Place a dry sterile gauze over the site of puncture and withdraw the needle. *Caution:* The tourniquet must be removed before withdrawing the needle from the vein, otherwise a hematoma may form at the puncture site.
19. Instruct the patient to open the hand and press the sterile gauze over the wound with the other hand for 3–5 minutes with the arm extended.
20. Remove the needle from the syringe and run blood down the side of the tube, using gentle pressure on the plunger to avoid foam or rupture of the cells.
21. If an anticoagulant is used, immediately invert the tube several times *gently* to mix the blood and anticoagulant completely.
22. Immediately after use, immerse the needle in *cold* tap water and draw the plunger back and forth several times to rinse out the blood before it coagulates in the equipment. Disassemble needle, syringe, and plunger and immerse in cold water.

DISCUSSION

1. Veins may be made more prominent and easier to enter by allowing the arm to hang down for 2 or 3 minutes, by massaging the blood vessel toward the body, and/or by smartly but lightly slapping the site of puncture.
2. Young and vigorous persons usually have elastic veins well filled with blood so that entrance into the vein is easy. Elderly or weak persons may have sclerosed veins which are hard to enter or the pressure may be so low that veins collapse under pressure of the needle or vigorous pulling of the plunger.
3. Occasionally the best vein is located on the hand, leg, or foot. These areas are more sensitive, however, and the veins usually less firmly anchored than those of the arm.
4. Rolling veins may be held by placing the thumb on the vein so that a 1– or 2–inch length of vein lies between the thumb and the puncture site. As soon as the vein is entered, the thumb is removed.
5. If difficulty is experienced in entering the vein or a hematoma commences to to form, release the tourniquet and promptly withdraw the needle and apply pressure to the wound.
6. Vigorous pulling on the plunger of the syringe may collapse the vein, produce hemolysis of the blood specimen or cause air to enter the syringe.
7. When repeated venipunctures have to be performed on one patient, it is ad-

visable to select different sites for blood withdrawal.

8. Vacuum syringes have recently come into current use in many hospitals.

9. Blood drawn by venipuncture is often stored for a period of time in vials or flasks until the analysis is made. The time interval of storage will vary. For this reason, certain general precautions must be followed in order to insure a valid analysis. Before withdrawing blood from its container, make sure the blood sample is thoroughly but gently mixed. Blood containers should be tightly corked at all times to prevent drying or contamination. When not in use, the blood specimen should be kept in the refrigerator. If blood counts are to be performed, the blood should be examined within 3 hours after collection. Platelet counts should not be performed on specimens treated with anticoagulant. Under no circumstances should blood taken for hematological examinations be stored overnight.

OBTAINING BLOOD SPECIMENS BY CAPILLARY PUNCTURE

Capillary puncture is one of the most common methods of collecting a blood sample (see fig. 2). It is easy, simple, and causes less pain and anxiety to the patient if it is correctly performed. It can be accomplished quickly and requires less skill than any other method of blood specimen collection. Careful mixing of the blood specimen is rarely necessary, and the cell distribution resembles that normally found in the circulating blood. Treatment of the blood sample with anticoagulant is not often required and this lessens the danger of cell distortion. Minimizing chances of cell distortion is especially important when blood is obtained for the purpose of making a differential count in a

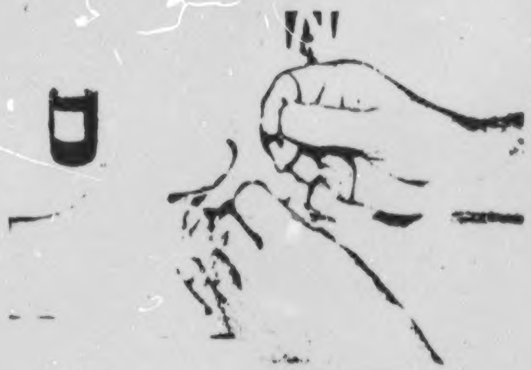


Figure 2—Continued. STEP 2—After allowing sufficient time for the circulation to equalize, puncture the site with a blood lancet. The puncture should be made with a quick firm stab.

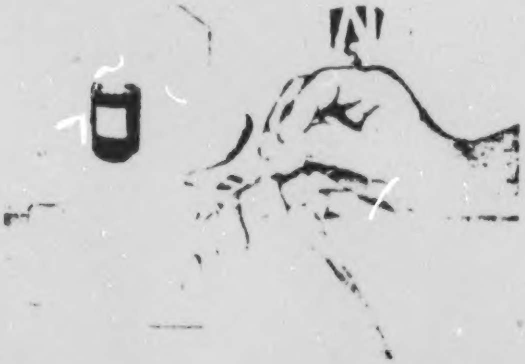


Figure 2. Obtaining Blood Specimen by Capillary Puncture. STEP 1—Rub the site of puncture with an alcohol soaked sponge to remove dirt and epithelial debris and to increase the amount of circulation of blood in the part.

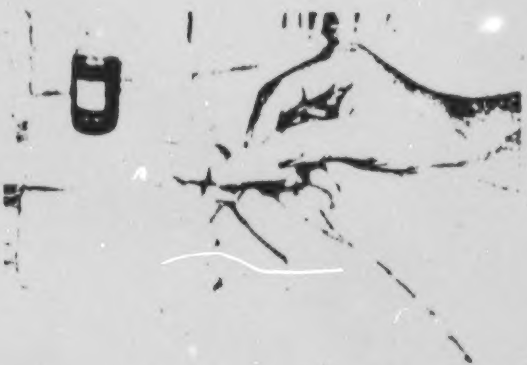


Figure 2—Continued. STEP 3—After withdrawing the lancet, a drop of blood will appear. The first drop of blood that appears is wiped away.

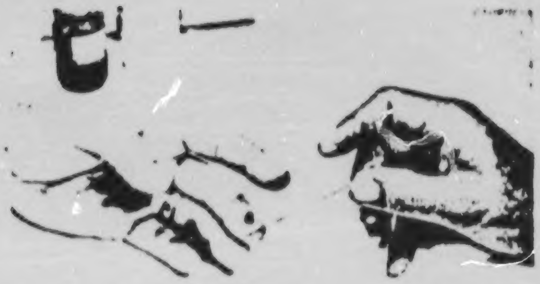


Figure 2—Continued. STEP 4—The second drop of blood is the one used for the test.

Figure 2—Continued. STEP 5—After the specimen has been taken, have the patient apply a sterile gauze pad to the puncture site until bleeding stops.

blood smear. Several different sites are suitable for capillary puncture. Because it is the most accessible, the palmar or lateral surface of the tip of the finger (preferably ring finger) is the most common site in adults. However, certain problems may be encountered such as heavy calloused areas or excessive tissue fluids (edema) that tend to result in nonrepresentative samples. The lobe of the ear may be used for capillary puncture. However, differences in cell concentration do occur when blood is obtained from this site, primarily because of higher lymphocyte concentrations in the ear lobe.

Because of the small amount of tissue on the fingers of infants, the preferred site is the heel or big toe. A modification of the normal technique that has proven quite satisfactory when working with infants is to make two incisions in a criss-cross fashion or "T."

Note: To be a valid report, work done on capillary blood must be from a FREE--FLOWING puncture wound.

APPARATUS

Gauze sponges, 2 x 2 in.
Blood lancet
Glass slides, capillary tubes, or other device to receive specimen.

REAGENTS

Alcohol, 70 percent, or other suitable disinfectant (2)

TECHNIQUE

1. The puncture site should be warm to assure good circulation of blood. If it is cold, apply warm water (38°–40° C.) for a few minutes. If blood is to be drawn from the ear, the edge of the lobe, not the flat side, should be punctured.
2. The site to be punctured is first rubbed with alcohol soaked gauze to remove dirt and epithelial debris, increase circulation and render the area reasonably sterile (fig. 2).
3. Allow sufficient time for the circulation to equalize.
4. While making a finger puncture apply gentle pressure to the finger to hold the skin taut. Hold the finger in one hand and the lancet in the other.
5. The puncture is practically painless if properly made with a sharp instrument. It should be made with a quick, firm jab, and 3–4 mm in depth.
6. Finger punctures should be made along the lateral aspect of the fingertip. More nerve endings are located on the fingerprint area of the fingers, therefore, more pain results from punctures in this area. Scars may also form in these sensitive areas, and difficulty may be encountered in puncturing a callous. All of these difficulties are eliminated by drawing the blood from the lateral rather than the ventral aspect of the finger.

7. The first drop of blood that appears is wiped away before specimens are taken.
8. The blood must *not* be squeezed out since this dilutes it with fluid from the tissues, thus altering the ratio of cellular elements to fluid, as well as the ratio of cellular elements to each other.
9. After the desired specimens have been collected, have the patient hold a sterile dry gauze over the wound until bleeding stops.

DISCUSSION

1. The "hemolet" is a very satisfactory disposable instrument for puncture of the skin. However, if this is not available a No. 6 Hagedorn needle or a No. 11 Bard-Parker blade is quite suitable. *Caution:* If any lancets are reused, they *must be* properly autoclaved and not merely soaked in alcohol. Because corks are easily contaminated, needles or blades inserted in corks should not be used.
2. Do not use the finger on a hand which has been hanging over the side of the bed as it is likely to be congested. Edematous or cyanotic areas should not be used.
3. The finger should be thoroughly dry *prior* to puncture; blood will not well up on a finger that is moist. Furthermore, the alcohol or other antiseptic used may coagulate the blood proteins causing cell clumping and erroneous values as well as dilute cell volumes. This will result in incorrect counts and differentials.

ANTICOAGULANTS

In obtaining blood by venipuncture the only reagents which are generally used are antiseptics and anticoagulants. The antiseptic is used to clean and sterilize the skin area over the site of venipuncture. The most common antiseptic is 70 percent ethyl alcohol. The anticoagulant used will depend on the analysis to be made and the preference of the technician.

Anticoagulants are used to prevent the coagulation of the blood specimen and the reagent employed should not be such that it brings about alteration of blood components. Unfortunately, many coagulants may alter cell structures as well as coagulation. In Army Medical laboratories the anticoagulants most often used are dried ammonium and potassium oxalate, and heparin.

The most commonly used anticoagulant is ammonium and potassium oxalate (Heller and Paul's double oxalate). This combination has an advantage over single oxalates alone because it does not cause shrinkage or enlargement of the red blood cells. Ammonium and potassium oxalate is preferred for treatment of blood to be tested for sedimentation rate and also for most other determinations. Ammonium oxalate cannot be used when tests for urea are to be carried out.

Heparin does not alter the size of the corpuscular components but care must be exercised in its use since it does not mix readily with blood and it may bring about the formation of tiny clots as well as enhance cellular disintegration.

The proper quantity of anticoagulant must be used for each specimen. If the volume of the specimen is 5 ml of blood, enough anticoagulant should be present in the container to prevent coagulation of that much blood and no more. An excess of anticoagulant will be present for example, if less than 5 ml of blood is introduced into a vial containing anticoagulant in amount sufficient for a 5 ml specimen. This can result in distortion of cells and hemolysis.

If the anticoagulant solution (oxalate) is added to vials and dried in an oven, great care must be taken to avoid temperatures above 80° C. as excess heat can change the anticoagulant. For example, oxalate will be converted to carbonates by prolonged exposure to temperatures above 80° C.

Chapter 4

ERYTHROCYTE STUDIES

The cells of the erythrocyte series are subject to qualitative and quantitative variations. Purely qualitative variations include differences in size, shape, internal structure, and type of hemoglobin. Quantitative variations include a decrease or increase in the numbers of erythrocytes. Either a qualitative or quantitative change in erythrocytes may affect body metabolism by impairing the oxygen-carrying capacity of the red blood cells. Anemia may result if impairment is severe. Adequate evaluation of the anemias is dependent upon basic studies of erythrocytes.

The determination of the amount of hemoglobin contained in a blood sample may be used to estimate the oxygen-carrying capacity of the blood. However, this determination must be correlated with an evaluation of the morphology of the erythrocytes. A rapid but rather crude evaluation of the size, shape, and hemoglobin content of the red cells may be obtained by microscopic examination of a stained blood smear. More objective studies, however, are necessary to evaluate the condition of erythrocytes in blood and to accurately measure qualitative variations which may occur in normal erythrocytes.

Among the most valuable laboratory procedures which have been devised for erythrocyte evaluation are the erythrocyte indices (i.e., index as to the average size of the erythrocytes, amount of hemoglobin, etc.). Calculation of these indices depends upon accurate erythrocyte count, volumetric hemoglobin determination and hematocrit determination. These indices and the determinations required to derive them will be discussed in the present chapter. The sedimentation rate, or rate of settling of red cells, reflects certain morphological characteristics of these cells. The hemotocrit determination is closely related to the sedimentation rate and will be dealt with in the present chapter. Qualitative tests of erythrocyte fragility and other volumetric determinations useful in evaluation of various anemias will also be presented.

ERYTHROCYTE DEVELOPMENT

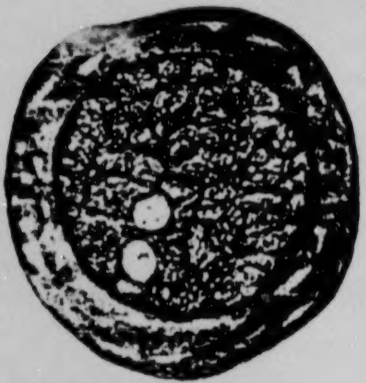
The erythrocytes, or red corpuscles, are the oxygen-carrying cells of the body. When mature, they are flattened to a biconcave disc shape, which facilitates the oxygen exchange of the cell.

The erythrocytes characteristically contain hemoglobin, an iron-porphyrin ring compound, which has the property of readily taking up and liberating oxygen. Without this hemoglobin-containing transport system, oxygen could not be carried in sufficient quantity by the blood to maintain body metabolism, and death would result.

Erythrocyte Maturation

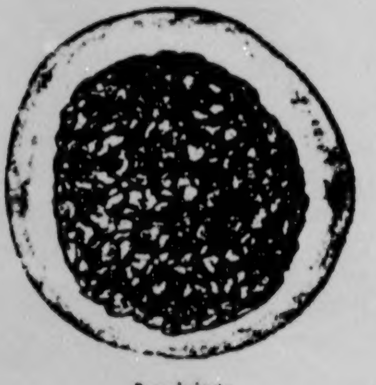
The term "erythropoiesis" refers to the production and development of the red blood cells. One must understand the normal to be able to recognize the abnormal. The stages in normal maturation of erythrocytes are enumerated below. Terms used are those recommended by the American Society of Clinical Pathologists and the American Medical Association. The cell descriptions are based on the appearance of well-prepared Wright's stained smears of normal blood or bone marrow.

- Rubriblast (pronormoblast)
- Prorubricyte (basophilic normoblast)
- Rubricyte (polychromatic normoblast)
- Metarubricyte (orthocromatic normoblast)
- Reticulocyte
- Erythrocyte



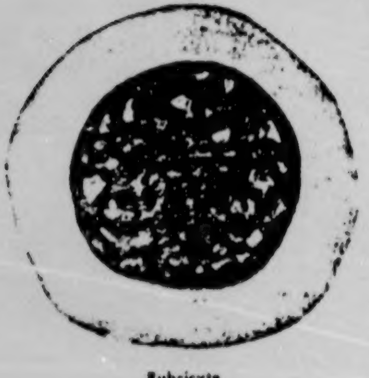
Rubriblast

Rubriblast. This cell is from 12–15 microns in diameter and has a large round purple nucleus which occupies most of the cell. The nuclear chromatin is fine, loose (spongy) and is arranged in a close mesh network. There are nucleoli present. The cytoplasm is deep blue, granule-free and limited to a thin rim around the nucleus. There is no evidence of hemoglobin formation.



Prorubricyte

Prorubricyte. This cell is about the same size as the rubriblast and contains a round or oval purple nucleus. The chromatin is *coarse* and clumped; nucleoli usually are not present. The cytoplasm of the prorubricyte is deep blue and cytoplasmic granules are not present. Hemoglobin cannot be demonstrated in this cell by routine stains.



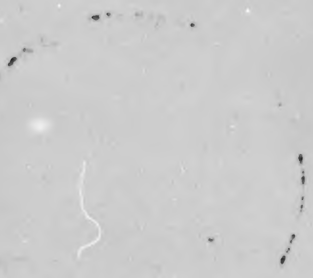
Rubricyte

Rubricyte. This cell is 12–15 microns in diameter with a round or oval, dark purple nucleus which is smaller than the nucleus in the prorubricyte state. The chromatin material is found in irregular clumps and no nucleoli are present. The cytoplasm of the rubricyte is blue-pink (polychromatic) the pink being due to the first appearance of hemoglobin. There [illegible] cytoplasmic granules.

Metarubricyte. This cell is smaller (8–10 microns in diameter) than the cells in the erythrocyte developmental series thus far discussed. The cell has a small round nucleus which is blue-black; the chromatin is usually uniformly dense. The cytoplasm appears as a wide band around the nucleus. The cytoplasm is buff-pink, with hemoglobin present in full amount. There are no granules present.

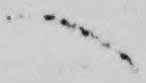
Metarubricyte

Reticulocyte. This cell is even smaller than the metarubricyte (8–9 microns in diameter). There is no nucleus in the cell. The remaining cytoplasm stains a bluish buff with Wright's stain, and there is no central light pallor as in the more mature cell. With supravital staining this cell will show light blue reticulum strands in the cytoplasm.



Reticulocyte

Erythrocyte. This is the mature red blood cell and normally averages 7–8 microns in diameter. The cell is biconcave and this causes a difference in the intensity of cytoplasmic staining. It has no nucleus. The cytoplasm of the periphery of the cell is buff-pink while the center zone is less intensely stained.



Erythrocyte

Variations in Erythrocytes

Erythropoiesis is regulated by the intake of substances to build the cells, the storage of these substances, and their proper utilization. When erythropoiesis occurs, both the cytoplasm and the nuclei of the cells grow at a certain rate. Individual differences in physiology and physical structure of the erythrocyte account for minor morphological changes so often encountered. In certain diseases, these morphological changes may vary to a greater extent. Only a few of the more common variations in erythrocytes will be considered here.

Probably the most striking of these variations is that observed in megaloblastic anemias, of which pernicious anemia is one example. Pernicious anemia is a disease considered to be due to a deficiency in vitamin B_{12}, and/or certain related growth factors. With this deficiency the erythrocytes do not mature normally and are generally larger than normal. The most notable characteristic of this abnormal maturation is a difference in the rates of maturation of the cytoplasm and the nucleus. The development of the nucleus is slower than that of the cytoplasm, so that in the more mature of the nucleated forms a spongy nucleus as well as an exceptionally large size may be observed. Such development is termed asynchronism. The mature cell is large (about 10 microns) and is termed a megalocyte. The younger cells of this series are named by adding the suffix "pernicious anemia type", or by changing the stem to the term "megalo", i.e., promegaloblast, basophilic megaloblast, etc.

Other factors sometimes produce morphological variations in red blood cells. For example, variations in the protein fraction due to certain genetic factors may result in changes in morphology to sickle shapes, elliptocytes or targets. Iron deficiencies sometimes cause rather striking changes characterized by erythrocytes which contain only a small thin rim of hemoglobin, e.g., hypochromia. Yet another variant causes the erythrocytes to be smaller and spherical rather than a biconclave disc.

Listed below are some of the more common variations which occur in erythrocytes. A brief description accompanies each variant. These variations are readily observed when blood smears are stained with Wright's stain. In addition to the variants discussed below, others also occur. Those not listed do not fall within the scope of this manual.

Crenated Erythrocytes. These are erythrocytes which have serrated or prickly outlines resulting from shrinkage of the cells. This may occur when the blood films dry too slowly and the surrounding plasma becomes hypertonic. This deformity is artificial and has no pathological significance except when such cells are found in spinal fluid.

Hypochromic Erythrocytes. This term refers to erythrocytes in which the normal central pallor is increased as a result of a decreased hemoglobin content. The condition in which extreme forms of hypochromia with only a narrow rim of hemoglobin remains at the periphery is called oligochromasia.

Polychromotophilia of Erythrocytes. This term refers to nonnucleated erythrocytes which still give a bluish appearance instead of the normal red color. This is due to the fact that the cytoplasm of these cells does not mature, resulting in the abnormal persistence of the basophilic cytoplasm of the earlier nucleated stages. Polychromatophilia denotes cellular immaturity.

Spherocytes. These are red cells which are spherical in shape, have a diameter smaller than normal, greater fragility, and a darker color (without central pallor) than normal erythrocytes. These cells are found in the hemolytic diseases and are particularly characteristic of congenital hemolytic jaundice, a mendelian hereditary disorder.

Anisocytes. This term is used to describe the presence of erythrocytes of several *different sizes* in the same blood specimen. It is a frequent finding in many types of anemia and is particularly pronounced in pernicious anemia where megalocytes and normocytes are found together. Cells smaller than 6 microns in diameter are spoken of as microcytes, while those larger than 9 microns are called macrocytes.

Poikilocytes. These are *irregularly-shaped* erythrocytes (pear-shaped, teardrop-shaped, burr cells, oval-shaped) which result from defective formation, mechanical division, deformation of fragile erythrocytes, or occur as an hereditary anomaly. These cells are encountered in pernicious anemia and in many other types of anemia.

Sickle Cells. The tendency to sickle cell formation is the characteristic feature of an hereditary anomaly which is encountered primarily in Negroes. This deformation is due to the presence of hemoglobin "S" and occurs in conditions of increased concentrations of carbon dioxide and reduced oxygen tension.

Target Cells. These red cells have colored centers and borders, separated by a pale ring, giving them a targetlike appearance. Large numbers of these cells are characteristic of Mediterranean anemia (Cooley's anemia) but may also occur in association with other anemias.

Howell-Jolly Bodies. These are nuclear remnants found in the erythrocytes of the blood in various anemias. They are round, dark violet in color, and are about 1 micron in diameter. Generally, only one Howell-Jolly body will be found in any one red cell. However, two or more may sometimes be present.

Cabot's Rings (Ring Bodies). These are bluish thread-like rings found in the red cells in the blood of patients with severe anemias. They are interpreted as remnants of the nuclear membrane and appear as ring or "figure-of-eight" structures. Usually only one such structure will be found in any one red cell.

Basophilic Stippling. Round, small, blue-purple granules of varying size in the cytoplasm of the red cell represent a condensation of the immature basophilic substance (see polychromatophilia) which normally disappears with maturity. This is known as basophilic stippling. It can be demonstrated by standard staining techniques in contrast to reticulocyte filaments which require a special stain. Stippling occurs in anemias and heavy metal poisoning (lead, zinc, silver, mercury, bismuth) and denotes immaturity of the cell.

Heinz-Ehrlich Bodies. These are small inclusions found primarily in those hemolytic anemias induced by toxins. They are round, refractile bodies inside the normocyte and are visible only in unfixed smears. It is thought that they are proteins which have been denatured and that they are an indication of erythrocyte injury.

Siderocytes. These are erythrocytes containing iron deposits. These deposits indicate an incomplete reduction of the iron from ferric to the ferrous state which is normally found in hemoglobin. Prussian blue stain must be used to readily demonstrate these cells.

RED BLOOD CELL COUNT

Principle (Ref: 2, 13, 33)

A sample of blood is diluted with a special isotonic solution in a micropipette. After adequate mixing, a portion of the sample is introduced into the counting chamber (hemacytometer). The red blood cells (erythrocytes) in a known volume are then counted.

APPARATUS

Venipuncture or finger puncture material
Thoma red blood cell diluting pipette, rubber hose, and mouthpiece
Hemacytometer
Hemacytometer coverglass

Note: The Thoma red cell diluting pipette is shown in figure 1 chapter 2. In the bulb is a small red bead. The capillary bore is graduated in units of 1,000, making the total volume of the bulb 100 units. The rubber tube and mouthpiece should be assembled and attached to the pipette just before it is to be used.

REAGENTS

Alcohol, ethyl, 70 percent (2)
Hayem's diluting fluid. (13)

TECHNIQUE

1. Draw well-mixed capillary or venous blood *exactly* to the 0.5 mark on the pipette using gentle suction on the mouthpiece. This blood column must be free of air bubbles. If capillary blood is used, wipe off the first drop of blood as it wells up on the finger. Touch the tip of the pipette to the second drop. Do not touch the pipette to the skin and *avoid squeezing* the finger to obtain blood.
2. Wipe the excess blood from the outside of the pipette to avoid transfer of cells to the diluting fluid.
3. *Immediately* draw Hayem's diluting fluid to the "101" mark, at the same time rotating the pipette between the thumb and forefinger to mix the specimen and diluent. Hold the pipette

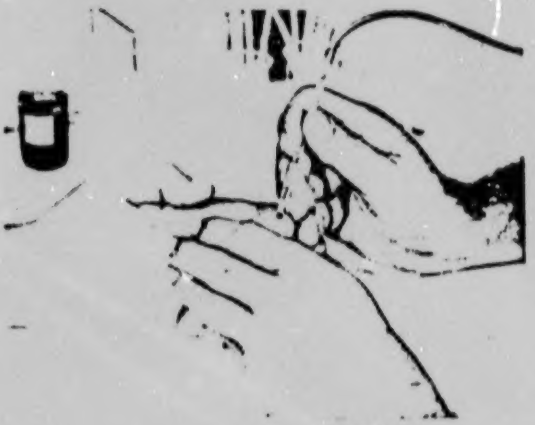


Figure 1. Obtaining and Processing Blood Specimen for Cell Count. STEP 1—After making finger puncture, wipe away first drop of blood. Bring tip of pipette into contact with second drop of blood and draw specimen to appropriate mark on the pipette tube.

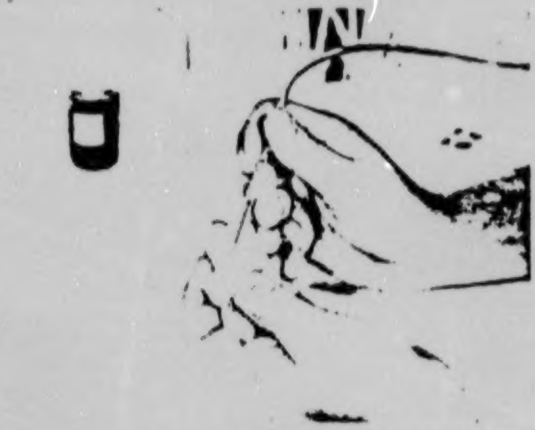


Figure 1—Continued. STEP 2—Wipe excess blood adhering to tip of pipette.

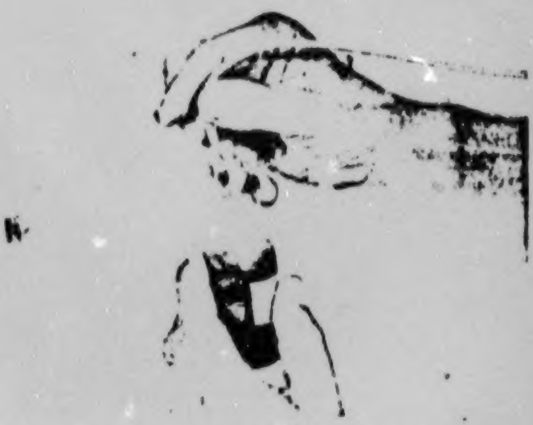


Figure 1—Continued. STEP 3—Insert pipette tip into appropriate type of diluting fluid and draw up fluid to proper marking on pipette tube.

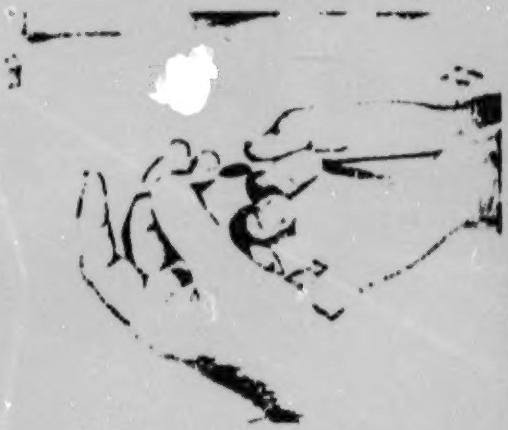


Figure 1—Continued. STEP 4—Holding index finger over pipette tip, remove rubber hose, then place thumb on opposite tip of pipette. Mix the contents of the pipette for about 10 seconds by holding the pipette on a horizontal plane, between the thumb and index finger of one hand and rotating in a "figure-of-eight."

upright to prevent air bubbles in the bulb.

4. Place a finger over the tip of the pipette and remove the rubber suction tubing.
5. Mix the contents of the pipette for about 10 seconds by holding the pipette on a horizontal plane, between the thumb and forefinger of one hand and rotating in a "figure-of-eight". A mechanical shaker may be used.
6. Place a clean hemacytometer coverglass on the counting chamber. Moistening the shoulders on which the coverglass rests aids in keeping a thin coverglass in place.
7. Mix the specimen in the pipette (as in step 5) for about 2 minutes to assure even distribution of cells.
8. Expel the unmixed and relatively cell-free fluid from the capillary portion of the pipette (usually two to three drops).
9. Place the forefinger over the top (short end) of the pipette, hold the pipette at a 45° angle and touch the pipette tip to the junction of the coverglass and the counting chamber (see fig. 2).
10. Allow the mixture to flow under the coverglass until the chamber is completely charged. Similarly fill the opposite chamber of the hemacytometer. If overflowing into the moat or air bubbles occur, clean and dry the chambers, remix the contents of the pipette, and refill both chambers.
11. Allow the cells to settle for about 3 minutes. Under low power magnification, focus on the ruled section of the counting chamber and locate the area for counting the red cells.
12. Observe for even distribution of cells.
13. Switch to high power magnification and locate the square in the upper left-hand corner (fig. 3).
14. Count all the red cells lying within this square and those touching the center line of the upper- and right-hand triple lines. The red cells which touch the left-hand and bottom center lines are not counted.

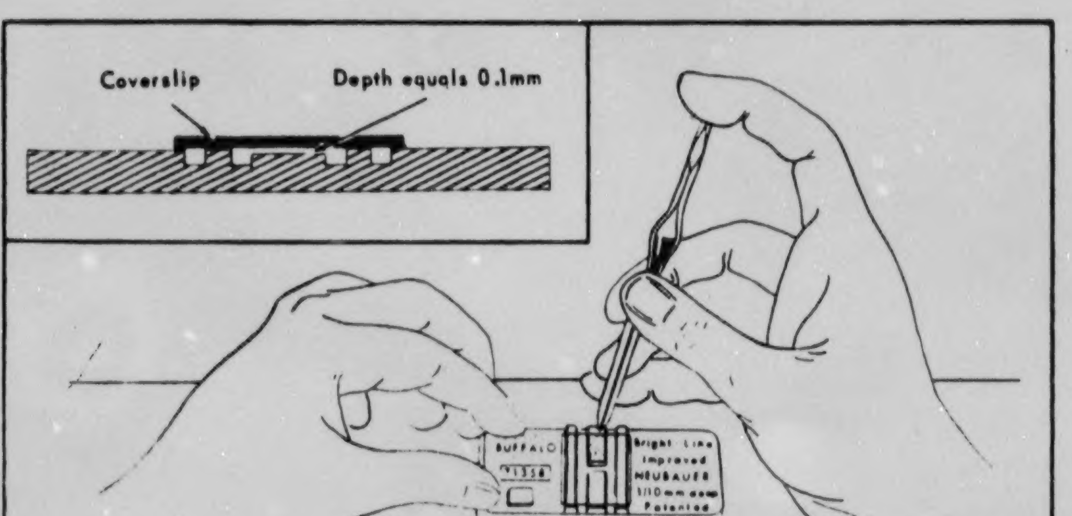


Figure 2 Method for charging hemacytometer Counting Chamber. Insert in cross-section view of the counting chamber.

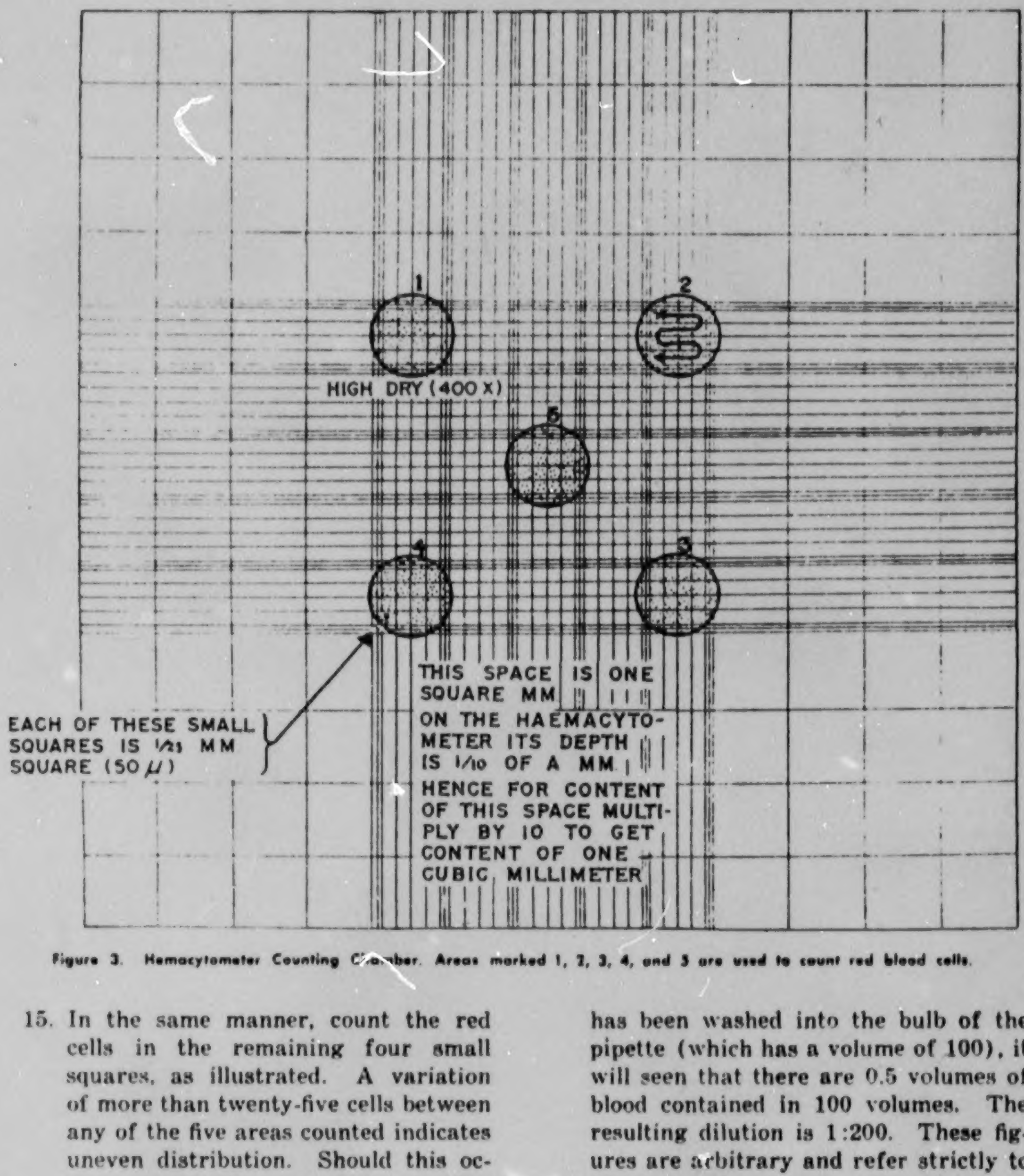

1
2
HIGH DRY (400 X)
5
4
3
THIS SPACE IS ONE SQUARE MM
ON THE HAEMACYTOMETER ITS DEPTH IS 1/10 OF A MM
HENCE FOR CONTENT OF THIS SPACE MULTIPLY BY 10 TO GET CONTENT OF ONE CUBIC MILLIMETER
EACH OF THESE SMALL SQUARES IS 1/25 MM SQUARE (50 µ)

Figure 3. Hemacytometer Counting Chamber. Areas marked 1, 2, 3, 4, and 5 are used to count red blood cells.

15. In the same manner, count the red cells in the remaining four small squares, as illustrated. A variation of more than twenty-five cells between any of the five areas counted indicates uneven distribution. Should this occur, repeat the procedure starting with step 7.
16. Count the second chamber in the same manner.

CALCULATIONS

1. Routinely, blood is drawn to the 0.5 mark and diluted to the 101 mark with RBC diluting fluid. Since all the blood has been washed into the bulb of the pipette (which has a volume of 100), it will seen that there are 0.5 volumes of blood contained in 100 volumes. The resulting dilution is 1:200. These figures are arbitrary and refer strictly to dilution and not to specific volumetric measurements.
2. The depth of the counting chamber is 0.1 mm; the area counted is 5/25 sq mm. In order to arrive at a final volume of 1 cmm, multiply the depth of the chamber by 10 and the area by 25/5 (or 5).

3. The formula is as follows:
   Average (of two chambers) number of RBC's counted
   x dilution (200) x depth (10) x area (5)
   EQUALS RBC's cmm.

EXAMPLE:

First Chamber Cells counted in each square	Second Chamber Cells counted in each square
110	99
100	102
95	87
115	95
90	107
510 RBC's counted	490 RBC's counted

Average of the two chamber counts

510
490
1000

$\frac{1000}{2} = 500$ RBC's counted

$500 \times 200 \times 10 \times 5 = 5{,}000{,}000$ RBC's/cmm

Note: The constant factors are depth, dilution, and area. Multiplying the latter factors, the result is 10,000. If the standard procedure described above is followed, the number of erythrocytes counted may be multiplied by 10,000 to obtain the total count per cmm.

NORMAL VALUES

Birth	4.8-7.1 million RBC's/cmm
Childhood:	3.8-5.4 million RBC's/cmm
Adult males:	4.6-6.2 million RBC's/cmm
Adult females:	4.2-5.4 million RBC's/cmm

DISCUSSION

1. Statistical analyses indicate that the probable minimum error of the red cell count is about 8 percent, even in the hands of trained technicians. In view of this high inherent error, determination of the packed red cell volume described elsewhere is recommended to replace the routine red blood cell count.
2. The minimum blood sample recommended for performing routine red blood cell counts is that obtained using one pipette and counting two chambers as outlined above. However, when the red blood cell count is to be used in the calculation of blood indices, greater accuracy is required. In this case, the procedure is to use two separate pipettes and fill two chambers from each pipette. The average count is then obtained and is used in the calculation of the blood indices.
3. When venous blood is used, it must be mixed properly with an anticoagulant at the time the blood is drawn and again just prior to pipetting to assure uniform distribution of cells. Vigorous mixing should be avoided since hemolysis may result.
4. If capillary blood is used, the technician must work rapidly following the puncture, otherwise coagulation of the specimen and settling of cells in the specimen are likely to begin before the blood is mixed with the diluent. This would result in a low cell count and/or a clogged pipette. Care must also be taken to minimize introduction of tissue fluids by "milking" the finger.
5. It is of the utmost importance that clean, dry diluting pipettes be used so that hemolysis is precluded.
6. Hayem's solution should not be more than 3 weeks old and should be filtered frequently. If the solution stands for any length of time, it becomes cloudy which makes it useless as a red blood cell diluting fluid.
7. Occasionally, blood "leaks" into the Hayem's solution from inserted pipettes and causes serious errors. Thus, routine filtration is necessary.
8. Pseudoagglutination of the red cells occurs in cases of abnormal plasma proteins (myeloma, Hodgkin's disease) or with an increase in cold agglutinins (viral pneumonia). This clumping may be prevented by adding 0.01 gm of gelatin to 100 ml of Hayem's solution. Heating of the Hayem's solution to 64° C. will counteract the effect of cold agglutinins without hemolyzing the red blood cells.
9. In polycythemia the number of red cells may be too great to permit accurate enumeration if a dilution of 1:200 is used. Blood is therefore drawn to the 0.3 instead of the 0.5

mark and the sample diluted to the 101 mark in the usual manner. The resulting dilution of 1:333 is used in the calculation.

10. In anemia the number of red cells may be too small to permit accurate enumeration if a dilution of 1:200 is used. Blood is therefore drawn to the 0.1 instead of the 0.5 mark and the sample diluted to the 101 mark in the usual manner. The resulting dilution of 1:100 is used in the calculation.
11. The use of anticoagulants which tend to shrink the cells will give high counts.

SOURCES OF ERROR

1. Obtaining blood specimen improperly.
2. Unclean or moist pipettes.
3. Using uncalibrated pipettes or pipettes with broken tips.
4. Failure to draw blood to but not beyond the 0.5 mark. Blood ascending beyond the mark deposits a thin film of cells even though the column is immediately brought down to the mark; this could produce an elevated count.
5. Inaccurate pipetting of diluting fluid. Fluid should be pipetted in a continuous motion to but never beyond the 101 mark. The washing backward and forward of blood and diluent in the pipette in an attempt to hit exactly the 101 mark overly dilutes the specimen.
6. Too slow manipulation following the withdrawal of the specimen thus allowing some of the blood specimen to coagulate.
7. Failure to mix the blood specimen and diluent thoroughly.
8. Improper charging of the counting chamber.
9. Uneven distribution of cells in the counting chamber.
10. Inaccuracy in counting the cells.
11. Use of diluent containing debris.

RETICULOCYTE COUNT

Principle (Ref: 13, 33)

In contrast to standard staining techniques, reticulocytes are stained supravitally. This is done by mixing equal amounts of whole blood and brilliant cresyl blue. A thin smear is then prepared from this mixture, dried, and counterstained with modified Wright's stain. The reticulocytes are reported as a percentage of mature red cells.

APPARATUS

Venipuncture or finger puncture material
Pipette, hemoglobin, Sahli-Hellige
Glass slides
Applicator sticks

REAGENTS

Brilliant cresyl blue stain (5)
Modified Wright's stain and buffer (37) (38)

TECHNIQUE

1. Place 40 cmm (2 Sahli hemoglobin pipettes full) of brilliant cresyl blue on a clean glass slide.

Note: A small test tube may be used in place of the glass slide.

2. Add an equal amount of capillary (or well-mixed venous) blood, mix well with an applicator stick, and allow to stand for 5 minutes. To prevent drying, the slide should be placed on a damp towel and covered with a Petri dish.
3. Touch a clean glass slide to the mixture, prepare a thin smear, allow it to dry, and counterstain with modified Wright's stain. Slightly alkaline stains give better results.
4. After the slide has dried, focus on the smear under low power magnification, and locate the thinner portion of the smear.
5. Cut a 5-mm hole in a circular piece of paper the size of a 5-cent piece. Place the disc in the ocular of the microscope to reduce the size of the field.

6. Switch to oil immersion magnification and count 200 red cells, including reticulocytes in each of five different areas on the smear (a total of 1000 red cells). Record the number of reticulocytes observed.

CALCULATION OF THE RETICULOCYTE COUNT

$$\frac{\text{Number of reticulocytes counted}}{10} = \text{percent reticulocytes}$$

NORMAL VALUES

Birth:	2.6-6.0 percent
Adults (both sexes):	0.5-1.5 percent

DISCUSSION

1. Reticulocytes are non-nucleated erythrocytes which exhibit blue reticulum strands within their cytoplasm when stained supravitally. When stained only with Wright's stain, they are buff-pink in color and larger and darker than erythrocytes.
2. Reticulocytes serve as an index of the activity of the bone marrow in blood regeneration. As such, these counts are of value in following anti-anemia therapy. Satisfactory response to therapy is evidenced by an increase of reticulocytes in the peripheral blood. Increased reticulocyte counts also occur whenever there is rapid bone marrow activity as in leukemia or blood regeneration associated with hemorrhage or hemolysis.
3. Several methods for staining and counting reticulocytes are in common use. Compared to the use of alcoholic solutions of dye, methods employing saline solutions of brilliant cresyl blue may give slightly higher values for reticulocytes. For comparative studies, the same method should be used throughout the work.
4. Precipitated stain is often confused with reticulum but may be recognized by its presence throughout the smear and apart from the red cells. Precipitation may be avoided by thoroughly washing the smear after staining and frequent filtering of the brilliant cresyl blue and Wright's stain.

HEMOGLOBIN DETERMINATIONS

The measurement of the hemoglobin in the blood may be the simplest method to determine the presence of anemia of polycythemia. It can be done quickly with a small amount of blood. The accuracy of the result, however, depends on the method used.

Because of wide variations in hemoglobin values of normal individuals and a variety of methods, it is impossible to assign a single universal "normal" value for hemoglobin. It is, therefore, recommended that hemoglobin values be expressed only in grams per 100 ml of whole blood. Significant variations in normal hemoglobin values exist between different age levels in children and between males and females in adults. A table of normal values which is applicable to the four methods of hemoglobin determination discussed follows.

Table I. Normal Values for Hemoglobin in Different Population Groups

Population group	Normal values in gm per 100 ml of whole blood
Infants at birth	18-27 gm
Childhood	10-15 gm
Adult males	14-17 gm
Adult females	12-16 gm

SPECTROPHOTOMETRIC METHODS OF HEMOGLOBIN DETERMINATION

Spectrophotometric methods of hemoglobin estimation (cyanmethemoglobin and oxyhemoglobin) eliminate the errors expressed in determinations made by the acid hematin techniques. Employing spectrophotometry, the probable error is less than 5 percent provided the pipettes used are correctly calibrated. Since the spectrophotometric methods are more accurate, these techniques should be used.

Spectrophotometer Calibration for Hemoglobin Determination

APPARATUS

Rubber stopper, No. 1
Spectrophotometer, Coleman Jr.
Cuvettes
Graph paper, semilogarithmic

REAGENTS

National Hemoglobin Standards (cyanmethemoglobin) (14)
Drabkin's solution (10)

TECHNIQUE

1. Cut a 5–mm slice from the top of a No. 1 rubber stopper and place it in the cuvette well to elevate the cuvette sufficiently for 5 ml of solution to span the light beam.
2. Pipette (using a suction bulb) 5 ml of Drabkin's solution to the "blank" cuvette, wipe it clean, and place it in the cuvette well containing the rubber support pad. Properly position the cuvette in the well of the spectrophotometer.
3. Set the spectrophotometer for 100 percent transmittance (%T) at a wavelength of 540 mu.
4. Wipe the hemoglobin standard cuvettes clean and, in succession, place the 5, 10, and 15 gm/100 ml standards in the cuvette well and record the percentage of light transmittance for each standard.
5. Plot %T against the grams of hemoglobin per 100 ml of blood on the semilogarithmic graph paper. Draw a line through the three points and prepare a table from this graph to show the hemoglobin values of all other percentage transmittances.
6. Recheck the calibration table once each week.

Note: The hemoglobin standards should be stored in the refrigerator until used. When used to calibrate for hemoglobin determinations, they should be allowed to warm up to room temperature to minimize condensation of moisture from the air on the cuvettes.

CYANMETHEMOGLOBIN METHOD

Principle

When a measured quantity of blood is diluted in Drabkin's solution, the hemoglobin is converted to cyanmethemoglobin. The hemoglobin content is then determined in a spectrophotometer.

APPARATUS

Venipuncture or finger puncture material
Pipette, hemoglobin, Sahli-Hellige
Pipette, volumetric, 5–ml
Spectrophotometer, Coleman Jr.
Cuvettes

REAGENT

Drabkin's solution (10)

TECHNIQUE

1. Pipette (using a suction bulb) exactly 5 ml of Drabkin's solution into a cuvette.

Note: Drabkin's solution contains poisonous cyanide and must be handled carefully.

2. Draw well-mixed venous (or capillary) blood exactly to the 20 cmm mark into a calibrated Sahli-Hellige hemoglobin pipette.
3. Wipe the excess blood from the outside of the pipette and add the contents of the pipette to the cuvette containing the Drabkin's solution.
4. Rinse the pipette several times with the Drabkin's solution to assure complete delivery of the blood sample.
5. At a wavelength of 540 mu, set the spectrophotometer to 100% T with the "blank" cuvette. The "blank" contains 5 ml of Drabkin's solution.
6. Substitute the cuvette containing the hemoglobin solution and read the percentage of light transmittance. Obtain the grams hemoglobin per 100 ml of whole blood by use of the graph prepared from the hemoglobin standards.

DISCUSSION

1. *Precautions in the use of cyanide:*
   a. Pipettes should be filled with a suction bulb, and *never* by application of suction by mouth.
   b. If cyanide powder should be spilled, immediately wipe up with a damp cloth and discard into a suitable container.
   c. Salts containing cyanide should be stored in a locked cabinet.
   d. Blood should be mixed with the cyanide solution by swirling.
   e. Discard all solutions containing cyanide into a free flow of water to minimize contact with other chemicals, especially acids, which will cause the release of poisonous fumes.
   f. Extreme care should be exercised in the preparation of Drabkin's solution Salts and solutions of cyanide are very poisonous and care should be taken to avoid getting them into the mouth or inhaling fumes. The ingestion of even small quantities of cyanide may result in death. *Always handle cyanide with great care!*
   g. Antidotes for cyanide with outlined procedures for administration should be posted in a conspicuous place in the laboratory. Speed in giving the antidote treatment is essential. Below is a suggested procedure which may be adopted:
      (1) Summon a doctor, but start treatment immediately!
      (2) Give amyl nitrite by inhalation (break capsule; hold under nose).
      (3) Open window for free circulation of air.
2. In view of the great degree of variability of the results of hemoglobin determinations using different techniques, it was recommended by the National Academy of Sciences, National Research Council that all laboratories adopt a uniform cyanmethemoglobin method of determination along with the National Hemoglobin Standards.
3. Cyanmethemoglobin is the most stable of the various hemoglobin pigments showing no evidence of deterioration after 6 years of storage in a refrigerator. The availability of prepared standards is a distinct advantage of this technique. All hemoglobin derivatives are readily converted to cyanmethemoglobin with the exception of sulfhemoglobin.
4. This method is highly accurate and is the most direct analysis available for total hemin or hemoglobin iron. Its disadvantage is the use of cyanide compounds which, if handled carefully, should present little hazard.
5. For accuracy in hemoglobin determinations, it is absolutely necessary that the spectrophotometer and Sahli-Hellige pipettes be accurately calibrated.
6. Venous samples give more constant values than capillary samples.
7. **National Hemoglobin Standards (cyanmethemoglobin) may be obtained from the Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington 12, D. C.**

SOURCES OF ERROR

1. Improperly calibrated pipettes.
2. Unclean cuvettes and pipettes.
3. Loose electrical connections and inconstant line voltage in the electrical current leads to the spectrophotometer.
4. Improper dilutions.
5. Improperly prepared Drabkin's solution.
6. Deteriorated Drabkin's solution. This solution should be stored in a brown bottle. With normal usage it remains stable at room temperature for months.

OXYHEMOGLOBIN METHOD

Principle (Ref: 2, 5, 13, 20)

Hemoglobin, in a measured quantity of blood, is converted to oxyhemoglobin by dilute ammonium hydroxide. The specimen is then placed in a cuvette and the hemoglobin content (in grams) is obtained spectrophotometrically.

APPARATUS

Venipuncture or finger puncture material
Pipette, hemoglobin, Sahli-Hellige
Pipette, serological, 10 ml
Test tube, 13 x 100 ml
Spectrophotometer, Coleman Jr.
Cuvettes

REAGENT

Ammonia water, 14 percent V/V (3)

TECHNIQUE

1. Pipette exactly 6 ml of distilled water into a 13 x 100 mm test tube.
2. Draw well-mixed venous or capillary blood exactly to the 20 cmm mark in a calibrated Sahli-Hellige hemoglobin pipette.
3. Wipe the excess blood from the outside of the pipette. Add the contents of the pipette to the tube containing the distilled water.
4. Rinse the pipette several times with the distilled water to assure complete delivery of the blood sample.
5. Mix the contents of the test tube to hemolyze the red blood cells. This tube may be stoppered and stored for several hours without introducing an error in the determination.
6. Transfer the contents of the test tube to a clean, dry cuvette. Add two drops of 14 percent ammonia water.
7. At a wavelength of 545 mu, set the spectrophotometer to 100% T with the "blank" cuvette. The "blank" contains 6 ml of distilled water and two drops of 14 percent ammonia water.
8. Place the cuvette into the spectrophotometer and obtain the hemoglobin content in grams/100 ml of the blood specimen from the direct reading scale or from a previously prepared calibration curve.

DISCUSSION

1. If the direct reading hemoglobin scale is not available, the standard percent transmission scale may be used. It will be necessary to standardize the spectrophotometer and to establish a curve of percent transmission in relation to grams of hemoglobin. Most standard texts carry detailed procedures for standardization of spectrophotometers and determination of hemoglobin curves.
2. On long standing, oxyhemoglobin is converted to alkaline hematin. This is a secondary reaction which is accelerated by excess alkalinity.
3. Venous samples give more constant values.
4. Though the "inactive" forms of hemoglobin (e.g., methemoglobin, carboxyhemoglobin, sulfhemoglobin) are not converted to oxyhemoglobin by the ammonium hydroxide, normally their absorption is so nearly identical that the *total content* of hemoglobin is determined as oxyhemoglobin.

SOURCES OF ERROR

1. Failure to read the test within 10

minutes after the ammonia water is added to the specimen.

2. Excess alkalinity.
3. Improperly calibrated pipettes.
4. Unclean cuvettes.
5. Loose electrical connection and inconstant line voltage in the electrical current leads to the spectrophotometer.

VISUAL METHODS OF HEMOGLOBIN DETERMINATION

Visual methods incorporate the use of acid to convert the hemoglobin in the blood to acid hematin. This conversion product is a colored mixture which can be visually compared with a color standard. Two methods are described below, Sahli-Hellige and Haden-Hausser.

SAHLI-HELLIGE METHOD

Principle (Ref: 2, 9, 13, 14)

Hemoglobin, in a measured quanity of blood, is converted to acid hematin by dilute hydrochloric acid. The acid hematin is then diluted in a calibrated tube until its color matches that of a set of glass standards. The concentration of hemoglobin in gms/100 ml is read at the level of the meniscus in the calibrated tube.

APPARATUS

Venipuncture or finger puncture material
Hemometer, Sahli-Hellige
Glass color standard
Measuring tube
Hemoglobin pipette (20 cmm), rubber hose and mouthpiece
Medicine dropper
Glass stirring rod

REAGENT

Hydrochloric acid, 0.1N (17)

TECHNIQUE

1. Place 0.1N hydrochloric acid to about the 2 gram mark in the calibrated measuring tube.
2. Draw well-mixed venous (or capillary) blood *exactly* to the 20 cmm mark in a hemoglobin pipette. Wipe the excess blood from the outside of the pipette.
3. Deliver the blood from the pipette into the fluid of the measuring tube. Rinse the pipette several times with the dilute acid to remove all traces of blood from the bore of the pipette.
4. Mix the blood thoroughly with the hydrochloric acid by gently tapping the tube with the finger, being careful not to cause bubbles. Dissolve all the blood to produce a uniform dark brown mixture.
5. Note the time and allow the mixture to stand for 10 minutes for color development (acid hematin formation).
6. Dilute the mixture in the measuring tube with distilled water until the color of the solution matches that of the glass standard. This is done by adding the water drop by drop, mixing the solution and comparing the colors after each addition. Hold a microscope lamp with a blue filter behind the color standards to provide the proper illumination.
7. When the color of the solution matches that of the glass standards, observe the level of the bottom of the meniscus. This level corresponds to the hemoglobin content as indicated on the gram scale etched on the side of the tube. The percentage value is etched on the opposite face of the tube. The percentage value should be ignored.
8. Report the hemoglobin content in grams 100 ml of whole blood.

DISCUSSION

1. The color of the acid hematin gradually increases in intensity, reaching its maximum at the end of 30 minutes. Since it is not practical to wait this long be-

fore making the reading, it is customary that the test be read at the end of 10 minutes when 95 percent of the color has developed. It is very important that the time interval be adhered to in order to insure duplicability of results.

2. Acid hematin is a suspension, not a true solution. The preparation is not optically clear and sedimentation may lead to serious errors. This is especially of consequence when dealing with the blood of leukemia cases.
3. White counts greater than 100,000/cmm will produce a turbid solution of acid hematin which will increase the hemoglobin report by 5-10 percent.
4. Fetal hemoglobin will not be converted to acid hematin.
5. There is an inherent error of 15–30 percent with visual techniques. Values obtained by the Sahli method should *never* be used in calculating blood indices.
6. Approximately 2-12 percent of the total hemoglobin content of the blood circulates as methemoglobin, carboxyhemoglobin and sulfhemoglobin which cannot be converted in acid solution to acid hematin.

SOURCES OF ERROR

1. Inaccurately calibrated pipettes and measuring tubes.
2. Inaccurate glass standards.
3. Failure to maintain normality of HC1 at 0.1N.
4. Unstable color of acid hematin.
5. Differences in persons in their ability to compare colors.
6. Improper light source.
7. Eye fatigue.
8. Failure to read the test at the end of 10 minutes.
9. Faulty and hasty readings.
10. Improperly cleaned instruments.
11. Failure to discard the first drop of blood from a capillary puncture.
12. Failure to accurately fill the pipette. The specimen should be aspirated only as far as the 20 cmm mark. If overly filled a residual film of erythrocytes will remain in the tube. This can produce a falsely elevated value.
13. Contaminated diluting fluid (change to fresh fluid often).
14. Failure to make the proper dilution.

HADEN-HAUSSER METHOD

Principle (Ref: 13)

A measured quantity of blood is treated with acid and set aside for 30 minutes. The hemoglobin is read by comparison against a set of color comparator standards.

APPARATUS

Haden-Hausser hemoglobinometer
White blood cell pipette

REAGENT

Hydrochloric acid, 0.1N (17)

TECHNIQUE

1. Draw blood exactly to the 0.5 mark on the white blood pipette. Draw 0.1N hydrochloric acid up to the 11.0 mark.
2. Mix by rotating and allow to stand for 30 minutes.
3. Shake the pipette and discard fluid in capillary part of pipette.
4. Discharge the contents of the pipette into the wedge-shaped channel of the comparator at the end of the cover-glass.
5. Move the comparator through the field until the proper color match is made.
    a. A series of illuminated rectangles separated by alternate dark spaces appear in the field of the reading lens.
    b. The lower half of each rectangle constitutes the color standard, while the upper half of each rectangle constitutes the dilution when the wedge-shaped chamber is filled with the acid hematin solution (see fig. 4).

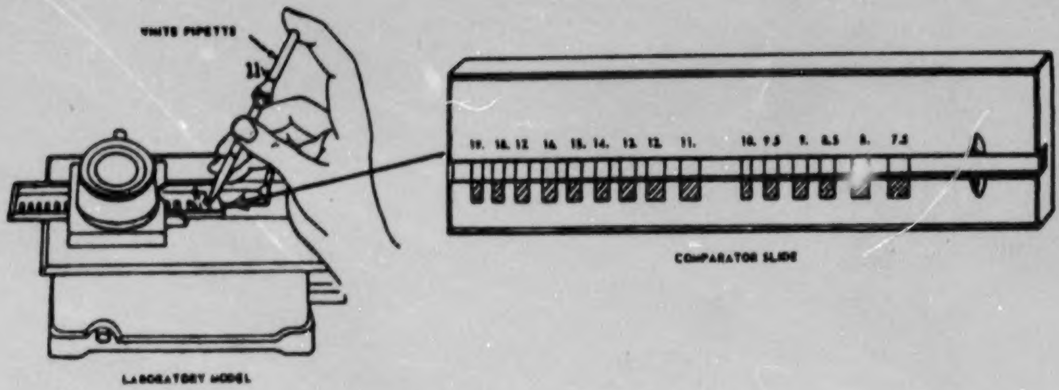


Figure 4. Haden-Hausser Apparatus for Hemoglobin Determination.

c. A thin, uniform film of the acid hematin will extend also by capillarity from the dilution chamber and cover the comparator color standard. This insures a light transmitting surface common to both the dilution channel and standard. The depth of the solution varies because of the wedge-shaped channel.

d. The scale, invisible during the process of color matching, is brought into the field by a shutter when the reading is made.

DISCUSSION

1. A convenience of the Haden-Hausser method is the use of the blood specimen in the white blood pipette to make a white blood count in conjunction with with the hemoglobin determination.
2. When the hemoglobin is below 7.5 gm, blood is drawn to the 1.0 mark on the pipette and the reading is divided by two. The technician should learn to estimate the hemoglobin as it appears in the pipette, and take a double amount when it is extremely low.

SOURCES OF ERROR

1. The color produced (acid hematin) is not stable.
2. There are inherent differences among persons in their ability to compare colors.
3. Inaccurate glass standards.
4. Inaccurately calibrated pipettes and measuring tubes.
5. Improperly cleaned instruments.
6. Improper light source.
7. Eye fatigue.
8. Failure to read the test at the end of 30 minutes.
9. Faulty and hasty readings.
10. Failure to discharge the first drop of blood from a capillary puncture.
11. Not accurately filling the pipette. The specimen should be aspirated only as far as the mark (0.5 mark on the white blood pipette). If overly filled a residual film of erythrocytes will remain in the tube. This can produce a falsely elevated value.
12. Pipetting of diluting fluid should be in a continuous motion to and never beyond the required mark. The washing action backward and forward of blood and diluent in the pipette while attempting to hit exactly the mark overly dilutes the specimen.
13. Contaminated diluting fluid (change to fresh fluid often).
14. Failure to make the proper dilution.

VARIATIONS IN HEMOGLOBIN

Hemoglobin exists in many forms. In general, the forms can be divided into two broad categories: variations in the iron or "heme" portion, and variations which occur in the globulin (globin) portion. Heme variations result from the combination of the iron (ferrous) component of blood with other chemicals. Normally, the iron combines with oxygen in a loose attachment known as oxyhemoglobin. In normal attachment with carbon dioxide it exists as reduced hemoglobin. Other combinations include: hemoglobin and carbon monoxide giving carboxyhemoglobin; hemoglobin and nitrites resulting in methemoglobin; and hemoglobin and inorganic sulfides producing sulfhemoglobin.

In practical application, certain hemoglobin combinations are not detectable by methods here described, i.e., sulfhemoglobin. Only oxyhemoglobin and reduced hemoglobin are capable of carrying oxygen. Detection of nonoxygen hemolglobin variants is accomplished principally by spectrophotometric or chemical analyses.

Prior to 1949, very little was known of the different configurations that the globin fraction of hemoglobin can produce. Since that time it has been found that globins possess dissimilarities in their chemical structure. These differences are based primarily on genetic differences.

Variations in the globin fraction of hemoglobin follow the general pattern of other proteins in that there is a multiplicity of variations. A few are:

Hemoglobin "A"—Adult hemoglobin.
Hemoglobin "F"—Fetal hemoglobin.
Hemoglobin "C"—Found associated with Mediterranean anemia.
Hemoglobin "S"—Found associated with sickle cell anemia.

Others ("D", "E", "H", etc.) are found in rare occurrence associated with several types of anemias. Their detection in the laboratory is primarily dependent upon electrophoresis, however, hemoglobin "S" may be detected because of the cell's characteristic shape when subjected to reduction of hemoglobin. The test demonstrating the sickle cell phenomenon is given below.

DEMONSTRATION OF THE SICKLE CELL PHENOMENON

Principle (Ref: 8, 13, 20)

Erythrocytes of persons with sickle cell anemia or trait will assume a sickle shape when the oxygen tension is lowered. This may be demonstrated by mixing a drop of blood with a reducing agent such as sodium metabisulfite.

APPARATUS

Glass slides
Coverglass
Medicine dropper
Applicator sticks

REAGENT

Sodium metabisulfite, 2 percent (33)

TECHNIQUE

1. Place one drop of capillary (or venous) blood on a clean glass slide.
2. Add one or two drops of 2 percent aqueous sodium metabisulfite and mix.
3. Place a coverglass on the preparation and express the excess blood by gently pressing the coverglass. The gentle pressure will produce a film thin enough to permit examination of individual red cells. It is not necessary to seal the preparation.
4. Observe immediately and at intervals of 15 and 30 minutes after preparation for signs of sickle cell formation.

DISCUSSION

1. The sickling phenomenon is a consequence of an inherited abnormal type of hemoglobin (hemoglobin "S"). The severity of the anemia as compared to the trait will vary with the proportion

of defective to normal hemoglobin. This abnormal hemoglobin can be identified electrophoretically.

2. When a fresh solution of sodium metabisulfite is used, positive cases should show 10 to 75 percent sickling within 15 minutes. There is a positive correlation between the degree of sickling and the severity of the disease.
3. Normal blood treated in the same manner shows no sickling.
4. The procedure of incubating a drop of whole blood in a sealed preparation may be unreliable. It is thought that the red cells may lose their ability to sickle before sufficient oxygen has been used up by cell metabolism.

HEMOGLOBIN ELECTROPHORESIS

One of the methods for separation of these different globins is by electrophoresis. The rate and the direction of the protein migration in an electrical medium is dependent upon the differential of the pH of the medium and the isoelectric point of the protein. Generally, the greater this difference the greater the rate of migration. Interpretation of the electrophoretic patterns is difficult since clear endpoints are often not obtained. Because of the variation in electrophoretic patterns and other technical problems, electrophoretic analysis is not detailed in this manual. Most electrophoretic analyses are performed at the larger research laboratories. To facilitate shipment of specimens to higher echelon research laboratories, the preparation of hemoglobin specimens for shipment is presented in the section which follows.

Preparation for Shipment of Specimens for Hemoglobin Electrophoresis

1. Withdraw 5 or 10 ml of blood into an oxalated tube.
2. Centrifuge for 10 minutes at 2,000 rpm.
3. Remove the plasma and wash the cells three times with 0.85 percent saline. Discard the saline after centrifugation.
4. To each volume of packed cells, add 1.5 volumes of distilled water and 0.5 volumes of toluene.
5. Shake vigorously until solution is bright red (5 minutes).
6. Chill in an ice bath for 30–60 minutes.
7. Centrifuge at 3,000 rpm for 30 minutes, preferably in a refrigerated centrifuge.
8. Pipette off toluene layer at top. Carefully remove or push aside the upper layer of lipid and stroma with an applicator stick.
9. Filter through Whatman #42 paper. The filtrate will be approximately 10 gm percent total hemoglobins.
10. Add 1–2 drops 1/10,000 merthiolate preservative.
11. Seal in a screw-cap tube. Pack in mailing container with *request slip* and a *descriptive but brief clinical history*.

Note: This lysis method is recommended for use in the field. Variations from this technique should be in accord with the instructions of the receiving laboratory.

HEMATOCRIT DETERMINATION (Packed Cell Volume)

The hematocrit is a test to determine the ratio of cells to fluid. This test is rapidly replacing the red cell count for general purposes since it is easier, quicker, and more accurate. Two methods are described here; the macromethod of Wintrobe and a micromethod.

MACROHEMATOCRIT METHOD (Wintrobe)

Principle (Ref: 13, 20, 33)

Whole blood is collected by venipuncture, mixed with an anticoagulant and introduced into a Wintrobe tube. The tube is then centrifuged to pack the cellular elements. The height of the packed red cell column is read and reported as a percentage of the total blood column. This percent value is the hematocrit or packed cell volume (PCV).

APPARATUS

Venipuncture material
Hematocrit tube (Wintrobe type)
Pipette, capillary
Test tube with anticoagulant

REAGENT

Ammonium and potassium oxalate (Heller and Paul's) (4)

TECHNIQUE

1. Draw 5 ml of blood by venipuncture, place the specimen in a test tube concontaining Heller and Paul's double oxalate and *mix thoroughly* by gently inverting the tube several times.
2. Fill the hematocrit tube to the 100 mm mark as indicated in the sedimentation rate technique described elsewhere. The blood column must be free of bubbles.
3. Place the hematocrit tube in a centrifuge and spin at 3,000 rpm for 30 minutes.
4. Note the height of the column of packed red cells at its junction with the "buffy coat". This level, as shown on the ascending scale etched on the tube, is the hematocrit and is reported in percentage (see fig. 5).

NORMAL VALUES

In the past, many different figures have been recorded as normal for PCV. These were caused by variations in the packing of cells due to centrifugation. Following are standard normal values which are applicable to both methods of PCV determination outlined here.

Birth:
44–64 percent (av. 54 percent)
Childhood:
34–41 percent (av. 38 percent)
Adult Males:
40–54 percent (av. 47 percent)
Adult Females:
38–47 percent (av. 42 percent)

DISCUSSION

1. This procedure is the most useful single index of the degree of anemia or polycythemia. It is the most accurate (2–4 percent error) of all hematological determinations. In contrast, the direct red blood cell chamber count has a percent error of 8–10%. The hematocrit is, therefore, preferable to the red blood cell count as a screening test for anemia. When feasible, this method should be substituted for the red blood cell count.
2. To allow for better separation of the cellular elements in the hematocrit, the tube should stand for at least 15 minutes prior to centrifugation. This separation is adequately accomplished if a 1-hour sedimentation rate precedes the hematocrit.
3. Above the column of packed red cells is a layer of white cells and thrombocytes called the "buffy coat". This layer is normally about 0.5 to 1.0 mm in thickness and serves as a rough guide of white cell and platelet numbers. It should not be substituted for the white cell count as variations in size of cells influence the number estimated.
4. The appearance of the plasma might suggest certain abnormalities. Normally, this fluid is clear and straw colored. A golden-yellow colored plasma might indicate the presence of jaundice while a creamy-opaque appearance would suggest an excess of fat in the blood (lipemia).
5. In normal blood centrifuged at 3,000 rpm for 30 minutes, trapped plasma comprises an average of 4.3 percent of the red cell column. This is reduced to

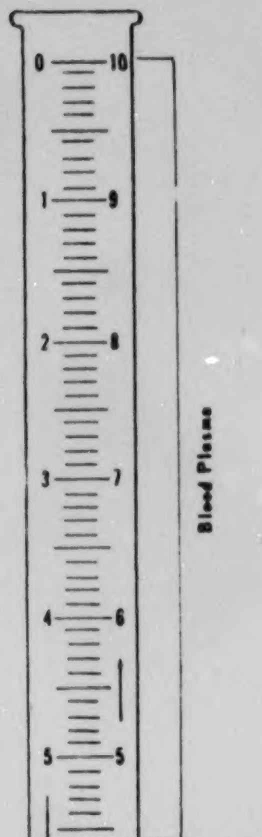


Figure 5 Wintrobe Sedimentation Tube

2.5 percent by centrifuging for 55 minutes. Anisocytosis and spherocytosis do not alter plasma trapping. Variation in the amount of plasma trapping is so small after centrifuging for 55 minutes, even with pathological bloods, that it is recommended over the 30-minute centrifugation when greater accuracy is desired.

SOURCES OF ERROR

1. Failure to use properly prepared Heller and Paul's double oxalate.
2. Failure to mix the specimen thoroughly. This might result in using an unrepresentative sample. Always remix the sample immediately prior to filling the tube.
3. Prolonged application of the tourniquet. If the tourniquet is left in place for more than 2 minutes, there will be concentration of the blood cells.
4. Improper centrifugation. The sample must be spun at least 30 minutes at 3,000 rpm. Neither the time nor the speed may be decreased without altering the results.
5. Misreading the red cell level by including the "buffy coat" will yield an increased result. The red cell layer may be more accurately differentiated from the leukocyte-platelet layer by using the microscope eyepiece as a magnifying glass when making the reading.

MICROHEMATOCRIT METHOD

Principle (Ref: 15, 19)

A heparinized capillary tube is filled with whole blood by gravity and capillarity to within 1 or 2 cm of the end. The unfilled end of the tube is then heat-sealed. Following centrifugation, the capillary tube is placed in a reading device and the hematocrit value determined.

APPARATUS

Finger puncture material
Capillary tubes, heparinized, (75 x 2 mm —"melting point tubes")
Centrifuge, microhematocrit
Reader, microhematocrit

REAGENT

Alcohol, ethyl, 70 percent (2)

TECHNIQUE

1. Cleanse the patient's finger with 70 percent alcohol.
2. Make a stab wound into the finger. Using a sterile gauze sponge, wipe off the first drop of blood as it wells up on the finger.
3. Touch a heparinized capillary tube to the second drop of blood and fill the tube to within 1 or 2 cm of the end.

Note: Blood will enter the capillary tube by gravity and capillarity.

4. Heat-seal the unfilled end of the capillary tube. This is done by holding the unfilled end of the tube at right angles to and just touching the outer margin of a microburner gas flame for about 3 seconds (fig. 4, Introduction).

   CAUTION: It is necessary that the tube be rotated constantly during the sealing process to assure that the inner base of the tube will be flat rather than tapered.

5. Place the capillary tube in one of the numbered slots in the centrifuge head in such a manner that the sealed end is *in contact* with the peripheral rim of the centrifuge head.

   CAUTION: Failure to have the capillary tube in contact with the rim might result in a shattered tube as the centrifuge speeds up.

6. Screw the flat centrifuge head cover in place.
7. Centrifuge the capillary tube at 10,000 rpm for 5 minutes.
8. Determine the hematocrit value with the aid of a microhematocrit reader.

Note: Since there are a variety of readers available, it is necessary that the technician carefully follows the directions of the manufacturer for the particular device utilized.

DISCUSSION

1. The microhematocrit technique is advantageous because of speed, and because only a small quantity of blood is necessary for the determination. An additional advantage is the ease with which this procedure is adapted to infants and small children. The microhematocrit technique requires only a simple capillary puncture whereas in the Wintrobe method venous blood must be used. Another advantage is the use of disposable capillary tubes.
2. If the microhematocrits cannot be read promptly, the capillary tubes must be properly identified and placed in a vertical position. Slanting of the cell layer will occur if tubes are left in a horizontal position form more than 30 minutes.

SOURCES OF ERROR

1. Improper sealing of the capillary tube.
2. Improper identification of capillary tubes.
3. Improper centrifugation.
4. Misreading the red cell level by including the "buffy coat."
5. Squeezing fingers, causing dilution of specimen with tissue fluid.

ERYTHROCYTE SEDIMENTATION RATE (ESR)

(Wintrobe and Landsberg)

Principle (Ref: 9, 13, 20, 33)

If blood, to which an anticoagulant has been added, is placed in a narrow tube, the blood cells settle out of the suspension, leaving clear plasma above them. In the erythrocyte sedimentation test, the distance that the red corpuscles have fallen within a given interval of time is measured.

APPARATUS

Venipuncture material
Hematocrit tube (Wintrobe type)
Pipette, capillary
Test tube with anticoagulant

REAGENT

Ammonium and potassium oxalate (Heller and Paul's) (4)

TECHNIQUE

1. Draw 5 ml of blood by venipuncture and place it in a test tube containing 0.5 ml of dried double oxalate.
2. Thoroughly mix the blood and anticoagulant by gently inverting the tube several times, being careful not to cause bubbles.
3. Draw the blood into the capillary pipette and fill the hematocrit tube to the "0" mark. This is done by inserting the capillary pipette to the bottom of the Wintrobe hematocrit tube, while holding it at an angle of 45°. As the tube is filled, slowly withdraw the pipette so that the tip is always just below the level of the blood. The blood column must be free of bubbles.
4. Place the filled hematocrit tube in a rack in an *exactly vertical* position and note the time and room temperature.
5. At the end of 1 hour, read the level to which the red cells have settled on the descending scale etched on the tube. Each mark equals 1.0 mm while each numbered mark equals 10 mm (1 cm). The figure obtained is reported in mm hour as the "uncorrected" erythrocyte sedimentation rate.
6. If the room temperature is more than 20°C. (68°F.), correct the sedimentation rate according to the temperature correction chart (fig. 6).
7. To correct sedimentation rate for temperature variation:
   a. First, follow the vertical line representing the temperature of the room to the horizontal line representing the sedimentation rate. Secondly, follow the nearest sloping line to the left hand side of the chart to obtain the corrected value at 20°C.
8. Additional correction of the erythrocyte sedimentation rate is necessary if the packed volume is less than normal (42 percent for women and 47 percent for men). Details for this type of correction are described following this method.

NORMAL VALUES

Males: 0–9 mm/hour (av. 3.7 mm/hour)
Females: 0–20 mm/hour (av. 9.6 mm/hour)

DISCUSSION

1. The sedimentation rate is a nonspecific test which suggests the possibility of a disease process and tissue damage in the body. It is not diagnostic but is extremely useful in following the course of some diseases.
2. The rate of settling of red blood cells depends on plasma and cellular factors. The former is of greater influence with fibrinogen having the greatest single effect upon increasing the erythrocyte sedimentation rate. Fibrinogen causes the red cells to aggregate in rouleaux formation which leads to more rapid cell settling because of a larger mass of cells exposed area.
3. In disease processes where there is an increase in fibrinogen and or globulin (e. g., rheumatic fever, arthritis, tuberculosis, etc.), the sedimentation rate

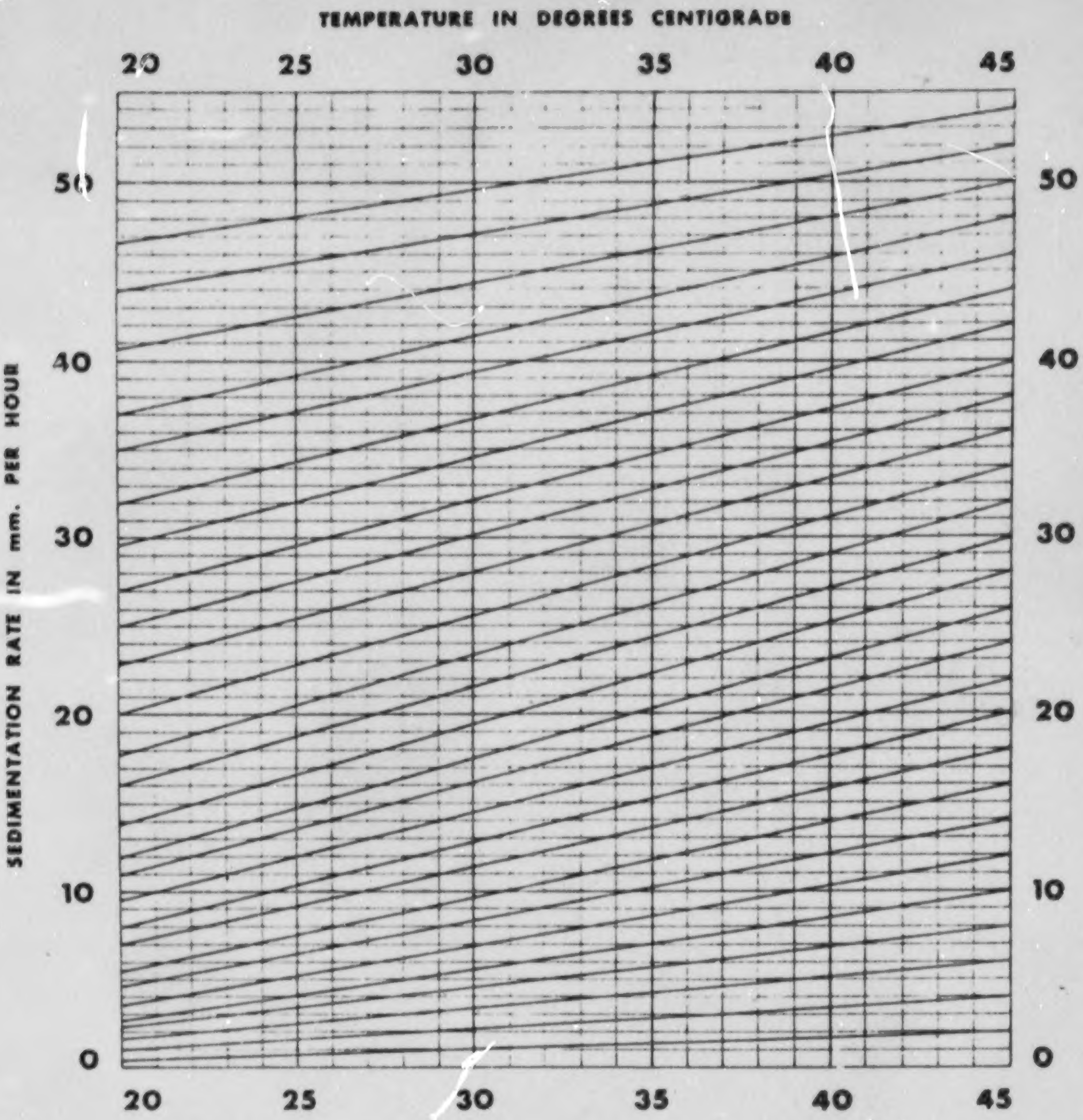


Figure 6. Variations in Sedimentation in mm Per Hour in Relation to Temperature.

may be elevated over long periods of time even though the disease activity may not manifest itself by fever or by leukocytic changes.

4. Whenever the number of red cells is less than normal (decreased hematocrit), there is less retardation of sedimentation by the erythrocytes themselves and they tend to settle faster. This apparent increase in the sedimentation rate, due to anemia, must be corrected.
5. The sedimentation rate is of little clinical value when the hematocrit exceeds 55 percent for the cells will not settle out readily.
6. Hypochromic, microcytic red cells tend to settle more slowly than normal cells, whereas macrocytic cells fall more

rapidly. An increase in reticulocytes and or bizarrely-shapped red cells will decrease sedimentation rate.

7. The ESR should correlate with C-reactive protein determination.

SOURCES OF ERROR

1. Improper preparation of the anticoagulant.
2. Improper proportion of blood to anticoagulant.
3. Not correcting for temperatures above 20°C.
4. Delay in performing the test.
5. Leaving the tourniquet on the patient's arm longer than 2 minutes.
6. Failure to mix the specimen properly before transferring it to the Wintrobe hematocrit tube.
7. Placing the hematocrit tube the slightest degree from the vertical.
8. Hemolysis of red cells.
9. Unclean capillary pipettes or Wintrobe tubes.
10. Reflux of blood above the "0" mark brought about by bubbles in the Wintrobe tube.

CORRECTION OF SEDIMENTATION RATE OF ANEMIC BLOOD

(Wintrobe)

If the packed cell volume is below 42 percent for women and 47 percent for men, the sedimentation rate must be corrected for anemia (using the chart shown as figure 7.) Report the "uncorrected" as well as the "corrected" sedimentation rate. In the chart, the mean normal of packed erythrocytes for men (47%) and for women (42%) are heavily outlined and the range of normal sedimentation is represented by a solid

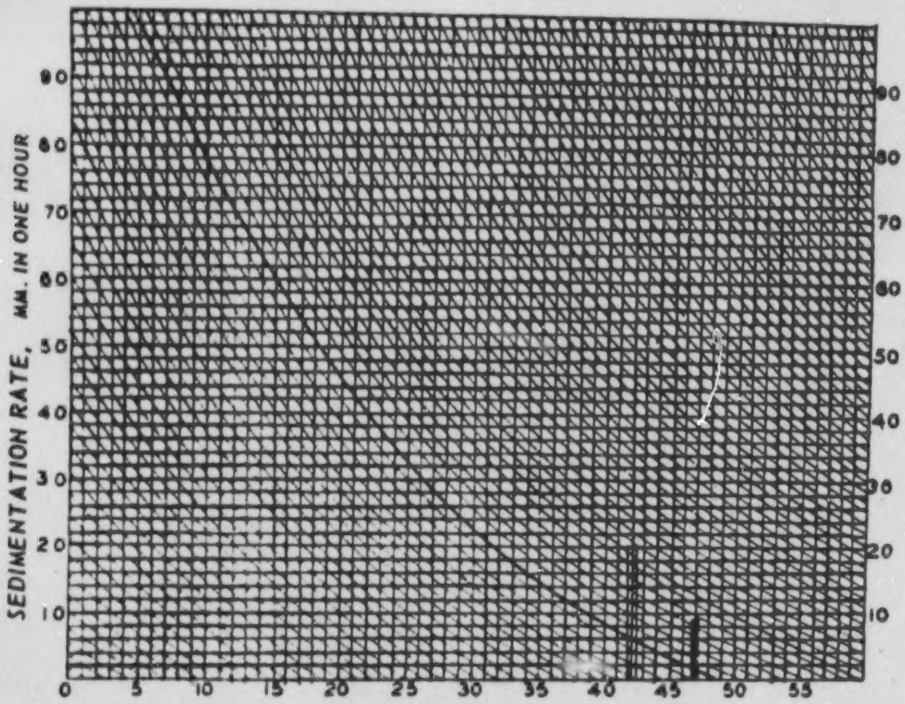


Figure 7. Chart for the Correction of Sedimentation Rate According to Volume of Packed Red Cells.

column for men and an open column for women. Directions for corrections of sedimentation rate according to the chart follow.

Note: Polycythemic blood should not be corrected using this chart.

1. Find the horizontal line corresponding to the patient's sedimentation rate and follow it across to the vertical line corresponding to the patient's packed cell volume.
2. Locate the curved line nearest to this point of intersection and follow it unti it crosses the dark line at 42% if the patient is a woman, or at 47% if th patient is a man.
3. At this point of intersection, read the value of the horizontal line which is the corrected sedimentation rate.

EXAMPLE:

Male Patient	
Uncorrected Sedimentation Rate	30 mm/hr.
Packed Cell Volume	35 percent
Corrected Sedimentation Rate	13 mm/hr.

ERYTHROCYTE INDICES

Principle (Ref: 33)

By using accurately determined red blood cell counts, hemoglobin and hematocrit values, the size and hemoglobin content of the average red cell in a given blood sample can be calculated. The values obtained are the erythrocyte indices and aid in the classification and study of anemias.

CALCULATION OF ERYTHROCYTE INDICES

1. Mean Corpuscular Volume (MCV)—The average volume of the individual red blood cell.

$$\frac{\text{Hematocrit (percent)} \times 10}{\text{Red cell count (in millions)}} = \text{cubic microns}$$

Example: Hematocrit 45 percent
Red count 5,000,000/cmm

$$\frac{45 \times 10}{5.0} = \text{cubic microns (normal)}$$

2. Mean Corpuscular Hemoglobin (MCH)—The average weight of hemoglobin of the individual red cell.

$$\frac{\text{Hemoglobin (gm/100 ml)} \times 10}{\text{Red cell count (in millions)}} = \text{micromicrograms}$$

Example: Hemoglobin 15 gm/100 ml
Red count 5,000,000/cmm

$$\frac{15 \times 10}{5.0} = 30 \text{ micromicrograms (normal)}$$

3. Mean Corpuscular Hemoglobin Concentration (MCHC)—The percent of hemoglobin in the average red cell.

$$\frac{\text{Hemoglobin (gm/100 ml)} \times 100}{\text{Hematocrit (percent)}} = \text{percent}$$

Example: Hemoglobin 15 gm/100 ml
Hematocrit 45 percent

$$\frac{15 \times 100}{45} = 33 \text{ percent (normal)}$$

NORMAL VALUES

Mean corpuscular volume: 82–92 cubic microns

Mean corpuscular hemoglobin: 27–31 micromicrograms

Mean corpuscular hemoglobin concentration: 32–36 percent

DISCUSSION

1. It should be emphasized that *precise* individual determinations must be done in order that the indices be reliable. The following procedures must be followed:
   a. Red cell count—two separate pipettes and 2–4 counting chambers.
   b. Hemoglobin — spectrophotometric analysis (NOT Sahli).
   c. Hematocrit — Wintrobe: centrifuge at 3,000 rpm for 55 minutes.
2. The appearance of the red cells on a stained smear should always be used as a check against the calculated blood indices. Repeated indices on a patient who has received no treatment should check within 5 percent.
3. The MCHC *cannot exceed* the normal value, since the red cells cannot be supersaturated with hemoglobin. The MCHC is the most valid of the indices since it does not require the red cell count in its derivation. It is a good index of iron deficiency.

4. Though the MCV and MCH are increased at birth and fall to low values during childhood, the MCHC is fairly constant for all ages.

ERYTHROCYTE FRAGILITY TESTS

The determination of the resistance or susceptibility of the red blood cells to hemolysis is of considerable significance in the evaluation of the anemias. In the osmotic fragility test, a zone of hemolysis is established. This reflects the ability of the red blood cell membrane to withstand variations in osmotic pressure. Other fragility tests measure the ability of the erythrocytes to withstand variations in pH while in solution; withstand mechanical trauma, as when shaken in a solution containing glass beads; or treatment with various enzymes such as trypsin. The more common fragility tests will be discussed.

ERYTHROCYTE OSMOTIC FRAGILITY

(Spectrophotometric Method)

Principle (Ref: 12, 13, 20, 21, 27, 33)

A specific amount of blood is introduced into a series of tubes containing varying concentrations of hypotonic salt solutions. The ability of the erythrocytes to resist hemolysis in such solutions is a measure of their osmotic fragility.

APPARATUS

Venipuncture material
Test tubes containing Heller and Paul's anticoagulant
Test tubes, 13 x 100 mm
Cuvettes
Pipettes, serological, 1 ml
Spectrophotometer, Coleman Jr.

REAGENT

Sodium chloride, 1 percent solution (31)
Ammonium and potassium oxalate (Heller and Paul's) (4)
Ammonia water (3)
Distilled water (pH 7.35 to 7.5)

TECHNIQUE

1. Arrange two series of 15 tubes in a rack and number as indicated in column 1, Table II.
2. Mark one series of tubes PATIENT and the other CONTROL.
3. Using a 1 ml serological pipette, deliver 1 percent sodium chloride into each series of tubes in amounts as shown in column 2.
4. Using a second 1 ml serological pipette, bring the volume of all tubes to 1.0 ml by adding distilled water in amounts indicated in column 3.
5. Shake all tubes to assure thorough mixing being careful not to cause bubbles.

Table II. Tube Set-up for Erythrocyte Osmotic Fragility Test

Column 1	Column 2	Column 3	Column 4
Tube number	Ml of 1 percent NaCl/tube	Ml of distilled water/tube	Final salt concentration in percent
100	1.00	0.00	1.00
75	0.75	0.25	0.75
70	0.70	0.30	0.70
66	0.66	0.34	0.66
62	0.62	0.38	0.62
58	0.58	0.42	0.58
54	0.54	0.46	0.54
50	0.50	0.50	0.50
46	0.46	0.54	0.46
42	0.42	0.58	0.42
38	0.38	0.62	0.38
34	0.34	0.66	0.34
30	0.30	0.70	0.30
26	0.26	0.74	0.26
0	0.00	1.00	0.00

6. The final concentration in each tube is shown in column 4.
7. Draw 5 ml of venous blood from the patient and deliver it into a test tube containing Heller and Paul's double oxalate anticoagulant. Mix thoroughly.
8. Oxygenate the blood thoroughly by gently shaking in a large container, and deliver exactly 0.1 ml of this blood into each tube of the PATIENT series.
9. Similarly, collect 5 ml of venous blood from a normal individual, mix with dry double oxalate and add, after oxygenating, *exactly* 0.1 ml to each tube of the CONTROL series.
10. Mix and set aside for 15 minutes. Centrifuge all tubes of both series at 1500 rpm for 5 minutes.
11. Deliver 9.5 ml of distilled water to 30 dry cuvettes marked as in steps 1 and 2.
12. Withdraw 0.5 ml samples of the supernatant solutions from each test tube and deliver into its corresponding cuvette.
13. Add 2 drops of ammonia water to the series of tubes marked CONTROL.
14. At a wavelength of 545 mu, set the spectrophotometer at 100 %T with a cuvette containing the solution from tube No. 100 (1 percent sodium chloride) as the "blank".
15. Read and record the optical density for each tube of the CONTROL series.
16. Repeat the procedure from step 13 for all tubes of the PATIENT series.
17. Calculate the percent hemolysis at each salt concentration for both series of tubes.
18. Plot the curves of the patient's and control's osmotic fragility using the percent hemolysis as the vertical ordinate and the salt concentration as the horizontal abscissa.

CALCULATION OF PERCENT HEMOLYSIS

$$\frac{\text{O.D. of a particular tube}}{\text{O.D. of tube No. 0 (100 percent hemolysis)}} \times 100 = \text{percent hemolysis}$$

Example:

O.D. of tube No. 50 = 0.43
O.D. of tube No. 0 = 0.86

$$\frac{0.43}{0.86} \times 100 = 50 \text{ percent hemolysis}$$

DISCUSSION

1. In hypotonic salt solutions, erythrocytes take up water, swell to a spheroid shape and burst. In congenital spherocytic anemia the red cells with defective structure more readily rupture at salt concentrations closer to isotonicity (0.85 percent). These cells thus show an increased osmotic fragility. In contrast, the flat or thin but otherwise normal red cells of hypochromic anemia show a decreased osmotic fragility and do not hemolyze until lower salt concentrations are reached.
2. When hemolysis begins beyond the range of the prepared solutions or when intermediate dilutions are desired, the additional dilutions may readily be prepared using the 1 percent sodium chloride stock solution.
3. In cases where the results of the fragility test are borderline, the following procedure is recommended to enhance any latent abnormality in fragility. Incubate samples of defibrinated blood (control and patient's) at 37°C. for 24 hours under sterile conditions and controlled pH (7.35–7.50). The test is then performed as described above.
4. Decreases in pH increase osmotic fragility. A 1:10 blood-saline dilution, as in this procedure, approximates a 7.4 pH value, which is satisfactory. In using greater blood-saline dilutions, especially prepared buffered salt solutions are employed.
5. This test may also be run visually, with some sacrifice of accuracy, by allowing the blood-saline dilutions to stand at 20°C. for 45 minutes. The tubes are then lightly centrifuged (1,000 rpm for 3 minutes) and observed for signs of initial and complete hemolysis. A slight pink coloration of the supernatant fluid indicates initial hemolysis and a clear

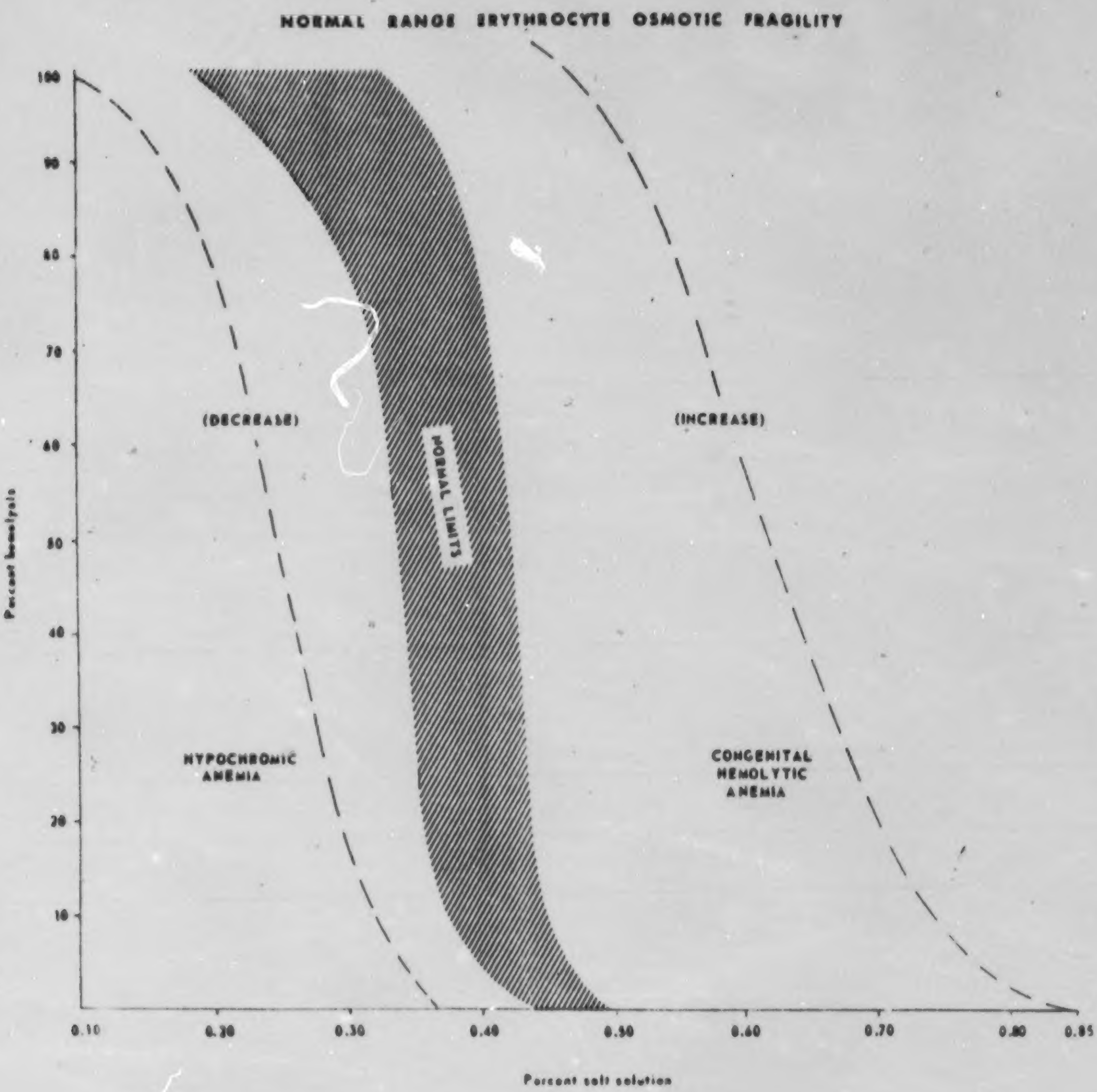


Figure 8. Relationship Between Erythrocyte Osmotic Fragility and Different Types of Anemia.

red solution, free of sediment, indicates complete hemolysis. The salt concentrations in these two tubes are noted and recorded. The control should always be reported along with results of patient's tubes.

6. The 1 percent sodium chloride solution is the negative control and should show no hemolysis, while the 0.00 percent sodium chloride is the positive control and should show 100 percent hemolysis. These two controls should always be run with each test.
7. To validate the results of this test, it is necessary to eliminate other causes of hemolysis, i.e., pH, heat, cold, mechanical damage to cells.

NORMALS

Initial hemolysis:	**0.44-0.40 percent**
Complete hemolysis:	**0.32-0.28 percent**

SOURCES OF ERROR

1. Inaccurate preparation and improper storage of the 1 percent salt solution. The salt must be chemically pure and weighed out when dry, e.g., after it has been dried in a sulfuric acid desiccator. The 1 percent solution must be kept in a glass-stoppered tightly closed bottle.
2. Inaccurate preparation of the various dilutions.
3. Poorly aerated (oxygenated) venous blood prior to use.
4. Variation in pH and temperature.
5. Rough handling of specimen causing hemolysis.

Ham Test for Erythrocyte Fragility (Ref: 13, 20)

This test is positive in paroxysmal nocturnal hemoglobinuria (PNH). Erythrocytes in this form of anemia lyse easily in slight variations in the pH (acid). In this test, the erythrocytes are subjected to pH values ranging from 6.5 to 7.0 at 37°C.

APPARATUS

Venipuncture equipment
Test tubes, Kahn

REAGENTS

Saline, 0.87 percent (28)
Hydrochloric acid solution, 0.33N (16)

TECHNIQUE

1. Carefully withdraw and defibrinate 10 ml of venous blood from the patient.
2. Carefully withdraw and defibrinate 10 ml of venous blood from a normal patient.
3. Centrifuge both blood specimens at 1,000 rpm for 10 minutes.
4. Separate serum and cells.
5. Wash the cells three times with normal saline, then prepare a 5 percent suspension of cells in normal saline.
6. Measure 1.0 ml of the 5 percent test cells into each of 4 tubes. Repeat this for the control normal specimen.
7. Acidify 1.9 ml of serum by adding 0.1 ml of 0.33N hydrochloric acid. Do this for both the test and control serums.
8. Into each of the tubes add test serum, control serum, acidified serum, and acidified test serum as follows:

Test	*Control*	*Add:*
1	1	1.0 ml test serum
2	2	1.0 ml control serum
3	3	1.0 ml acidified test serum
4	4	1.0 ml acidified control serum

9. Mix all eight tubes and incubate for 1 hour at 37°C. Shake every 10 minutes.
10. Centrifuge at the end of the 1-hour period and examine for hemolysis.

DISCUSSION

1. With a positive test the tubes containing acidified sera and patient's cells should show considerable hemolysis.
2. Normally no tubes should show hemolysis.
3. Occasionally, tubes with unacidified sera and patient's cells may show moderate hemolysis.
4. A false positive test is sometimes seen in congenital spherocytic anemia.
5. If congential spherocytic anemia is suspected, the test should be repeated, using acidified serum previously inactivated at 56°C. for 30 minutes.
6. Since erythrocytes of PNH require complement for hemolysis, the modified test (item 5 above) will be negative in PNH and will remain positive in spherocytosis.

SEROLOGICAL EXAMINATIONS

Some disease conditions manifest themselves by production of antibodies which can destroy the effectiveness of the erythrocytes. This destruction of function is primarily through hemolysis. The presence of these antibodies can be determined by tests for erythrocyte agglutinins and/or hemolysis.

Coombs Test

The Coombs test is a serological test used to detect the presence of incomplete antibodies. By themselves these antibodies are incapable of causing a direct erythrocytic agglutination. They do, however, react with antigen on the surfaces of the erythrocytes, thus coating or sensitizing these cells. The incomplete antibody coated cells then agglutinate after exposure to anti-human globulin antibodies (Coombs serum). Detailed procedures for both the direct and indirect test may be found in TM 8-227-3, "Laboratory Procedures in Blood Banking and Immunohematology".

SCREENING TEST FOR COLD AND WARM AGGLUTININS AND HEMOLYSINS AT pH 7.4

Principle (Ref: 33)

Some agglutinins that can also cause hemolysis may react at refrigerator temperature (3–4 C.) and not at 37°C. Other such antibodies may react at 37°C. Normal blood group O cells are subjected to the patient's serum and then incubated at the above temperatures and hemolysis and agglutination noted. Adequate controls should be run with this test.

APPARATUS

Serological tubes

TECHNIQUE

Add 1 ml of whole defibrinated blood to each of three tubes.

Tube 1

a. Centrifuge at 1,000 rpm for 5 minutes.
b. Examine the supernatant fluid for hemolysis.

Tube 2

a. Chill in ice bath for 20 minutes. Examine microscopically for cold agglutination.
b. Incubate for 1 hour at 37°C. Examine for agglutination. Spin at 1,000 rpm for 5 minutes and examine for hemolysis.

Tube 3

a. Incubate at 37°C. for 1 hour. Examine the erythrocytes for agglutination.
b. Centrifuge at 1,000 rpm for 5 minutes and examine for hemolysis.

DISCUSSION

1. Hemolysis in all three tubes is of no significance and is due to trauma to the erythrocytes.
2. Hemolysis in tubes 2 and 3 is due to a warm hemolysin and is seen in acquired hemolytic anemia.
3. Hemolysis in the second tube is due to a cold hemolysin. Agglutination in tube 2 is due to cold agglutinin. Agglutination in tubes 2 and 3 after heating is due to a warm agglutinin.

SCREENING TEST FOR WARM AGGLUTININS AND HEMOLYSINS AT AN ACID pH (Ref: 33)

APPARATUS

Serological tubes
Pipettes

REAGENT

Hydrochloric acid solution, 0.33N (16)

TECHNIQUE

1. Defibrinate 10 ml of blood. At the same time run a sample control of the same blood group.
2. Pipette off 5 ml of blood. Centrifuge at 1,000 rpm for 10 minutes and remove 3 ml of serum.
3. Pipette 0.1 ml of 0.33N hydrochloric acid solution into a small tube.
4. Add 1.8 ml of serum obtained above, wait 3 minutes, then mix.
5. Add 0.1 ml of whole defibrinated blood and gently mix.
6. Transfer 1.0 ml of the mixture to a second tube.
7. Centrifuge the first tube at 1,000 rpm for 5 minutes and examine for hemolysis.
8. Incubate the second tube for 1 hour at 37°C. Examine for agglutination and hemolysis.

DISCUSSION

1. Hemolysis of blood in the incubated tube and not in the control indicates one of two mechanisms:
   a. Abnormal RBC's are hemolyzed at an acid pH in normal serum. This is seen in paroxysmal nocturnal hemoglobinuria.
   b. A hemolysin, active at an acid pH is present in serum. This is seen in acquired hemolytic anemia. Agglutination of the RBC's indicates the presence of a warm agglutinin in serum.

ABNORMALITIES IN ERYTHROCYTE PRODUCTION AND DESTRUCTION

The number of red blood cells in the body of an adult is fairly constant. The total number or volume of erythrocytes can be measured by employing special tests. Knowledge of the total red blood cell volume and also total plasma volume may be of value to the physician in determining the amount of replacement blood or other intravenous fluids to be given to certain patients.

The red blood count based on the total erythrocyte volume may be subject to a slight, even hourly, variation. In the normal individual it remains essentially the same throughout adult life. The rate of destruction of the aged and worn out cells is approximately the same as the rate of production of cells. If the production-destruction ratio is not in equilibrium, either more or fewer red cells than normal will result. If the number of red cells is increased, the condition is known as polycythemia; if it is decreased, the condition is known as anemia.

Blood Volume Determinations

Blood volume determinations usually are carried out by introducing a foreign substance into the circulatory system and measuring its dilution after a given interval of time. One of the most practical methods is the injection of Evans Blue, T-1824. This is rapidly bound to the serum albumin, thus minimizing its elimination from the blood stream. Twenty minutes' time is allowed for the dye to mix in the circulating blood and samples are taken and compared in a photoelectric colorimeter. From the dilution of the dye, the circulating plasma volume is calculated. The blood volume is then determined from the hematocrit. The recent introduction of radioisotopes has made available a more accurate method of evaluating blood volumes. Radioactive iron, phosphorous, chromium, and radioiodinated serum albumin have all been successfully utilized.

Polycythemia

There are three types of polycythemia. The most common is known as relative polycythemia. It results from such conditions as increases in blood cell concentrations from plasma loss as in burns, dehydration from excessive vomiting, diarrhea, or sweating. The second most common form of polycythemia is known as secondary polycythemia. It is a result of some know condition, and is usually considered a response to anoxia. Secondary polycythemia may result from prolonged increase in altitude, anoxia due to congenital heart diseases, or chronic exposure to those chemicals which inactivate the oxygen carrying potential of hemoglobin, i.e., nitrate and carbon monoxide. The final type of polycythemia is known as polycythemia vera, a condition the cause of which is unknown. Polycythemia vera is characterized by a widespread stimulation of the entire bone marrow, with overproduction involving granulocytes, and platelets as well as erythrocytes. There is also an increase in the blood proteins, and an increase in the total blood volume.

Anemia

Anemia may be due to a decrease in the quantity or alteration in quality of the erythrocytes. Anemia is a symptom and not a disease and the classifiication of the anemias is difficult and complex. From the laboratory technician's standpoint, the classification of anemias is basically concerned with size, shape, and color of the red cells. The normal red cell is 7–8 microns. Variation in size of cells is called anisocytosis. Large cells are called macrocytes and the cells which are smaller than normal cells are called microcytes.

Cells exhibiting variation in shape from the normal biconcave disc are known as poikilocytes. Poikilocytes may be sickle-shaped cells, teardrop-shape, elliptocytes, target cells (bull's-eye-shaped) and many other forms. Crenated forms are not true poikilocytes but are artifacts caused by slow drying of the smear.

Normal erythrocytes are buff-colored due to hemoglobin content and are called normochromic. A cell that has only a small rim of color is said to be hypochromic. Hyperchromic or excessively colored cells do not exist because the erythrocytes do not exceed more than 33-36 percent hemoglobin, the normal amount.

On the basis of pathogenesis, the anemias may be classified into those due to deficient hemopoietic maturation factors, the hemoglobin deficiency anemias, and those due to deficiency in numbers of red cells. Normal hematopoiesis depends upon factors from the diet; intrinsic factor or vitamin B_{12} and folic acid; gastric absorption and liver storage factors. Deficiencies in any or all of these factors will result in the megaloblastic maturation typical of pernicious anemia and sprue.

Inherited protein abnormalities of hemoglobin have been discussed earlier in this chapter. These cause definite anemia conditions such as sickle cell anemia and Mediterranean anemia. Sickle cell anemia is characterized by the typical sickle-shaped cell while Mediterranean anemia exhibits target cells. Acquired abnormal hemoglobin deficiency may be caused from endocrine disorders, infection, poisons, or by radiation.

Hemorrhage or hemolysis may cause red cells to lose their ability to perform normal function. Chronic hemorrhage may not cause anemia as the system frequently compensates for the small loss. If the loss of blood exceeds production, an iron deficiency anemia may result. This will cause the red blood cells to be smaller than normal, a condition known as microcytosis. Normally, in addition to chronic blood loss, this hypochromic microcytic anemia may result from a poor diet, malabsorption, or poor iron utilization. With acute hemorrhage, the blood loss is so great that the body does not have ample time to compensate for the loss.

Hemolysis causes a shortened cellular survival time and results in the liberation of hemoglobin in the body. This brings about an increase in the pigment products beyond the body's capability to accommodate the load. Thus, there may be an increase in the serum bilirubin, urine urobilinogen, and stercobilinogen (fecal urobilinogen). This free hemoglobin which is released affects the body's metabolism by impairment of the oxygen-carrying capacity of the red blood cells. Such an anemia is logically called hemolytic anemia. Congenital hemolytic anemia is either caused by abnormal cellular stroma (elliptocytes, spherocytes) or abnormal hemoglobins ("C," "S," etc.) or both.

Acquired hemolytic anemia may be caused by bacterial or parasitic infections such as *Clostridium welchii* or malaria; chemicals or drugs such as arsenic, lead, and sulfonamides or from extensive burns.

Conditions causing a weakening of the cellular membrane result in hemolysis. Such conditions are associated with immune antibodies and the like found in cold and warm hemolysins, and incomplete antibodies of the ABO and the Rh categories. Special tests are required for the detection of the cause of hemolysis in this area. Such tests are the Coombs test, tests for cold and warm hemolysins and agglutins, and Ham's test for paroxysmal nocturnal hemoglobinuria..

Occasionally, the first indication of the presence of a hemolytic type of anemia may be the presence of erythrophagocytosis, for antibodies that can cause hemolysis can also stimulate erythrophagocytosis.

Quite often an increased demand on the hematopoietic system due to excessive loss of blood will result in the release of premature and atypical forms of erythrocytes into the circulation. These premature forms are the reticulocytic and normoblastic stages. Atypical forms are those in which the rate of development of the nucleus and the cytoplasm vary from the normal.

The most common of these forms is the non-nucleated erythrocyte which has a fine basophilic substance diffused throughout the cytoplasm. These are called polychromatic erythrocytes. Other forms result from the deposition of the nuclear material into configurations such as Cabot's rings and Howell-Jolly bodies. Heavy metals such as lead, zinc, silver, mercury, and bismuth cause additional atypical forms. The heavy metals precipitate a fine granular basophilic substance, which normally disappears upon maturity of the cells. This is called basophilic stippling.

Evaluation of the Anemias

Studies of the anemias are based on the symptoms of the disease and the type of red cells produced. Blood indices and reticulocyte counts are helpful in evaluation of the different forms. Fragility tests determine the degree of elasticity or strength of the cell membrane and cellular morphology studies are further aids to evaluation. The erythrocyte sedimentation rate and PCV are studies to evaluate the cellular suspension. A few of the more common types of anemias are listed below.

Microcytic Hypochromic Anemia. This is a common anemia characterized by red cells smaller than normal size and reduced hemoglobin content. Hypochromia is seen as an exaggeration of the central pallor of the erythrocytes. This is an iron deficiency type of anemia which develops as a result of poor diet, malabsorption or poor utilization of iron, or chronic blood loss.

Erythroblastosis Fetalis. This is a hemolytic anemia occuring in the newborn which is usually the result of Rh incompatibility and is characterized by normoblastic hyperplasia of the bone marrow and the presence, in peripheral blood, of large numbers of normoblasts and reticulocytes.

Acute Hemolytic Anemia. This condition is characterized by symptoms of rapid blood destruction. It may be the result of malaria, septicemia, bartonellosis, a variety of chemical agents, extensive burns, snake venoms, or circulating antibodies. The blood picture is usually that of a normocytic anemia with a high reticulocyte count, varying numbers of other immature erythrocytes (polychromatophilic cells, or normoblasts) and many anisocytes. White blood cells and platelets may also be increased. The osmotic erythrocyte fragility test is usually normal. The bone marrow is hyperplastic.

Congenital Hemolytic Jaundice. This is a chronic, hereditary, hemolytic anemia characterized by an increased erythrocyte osmotic fragility, many small spheroid-shaped erythrocytes and reticulocytosis. Polychromatophilia and normoblasts may also appear in the peripheral blood. The MCV is normal but the red cells are increased in thickness.

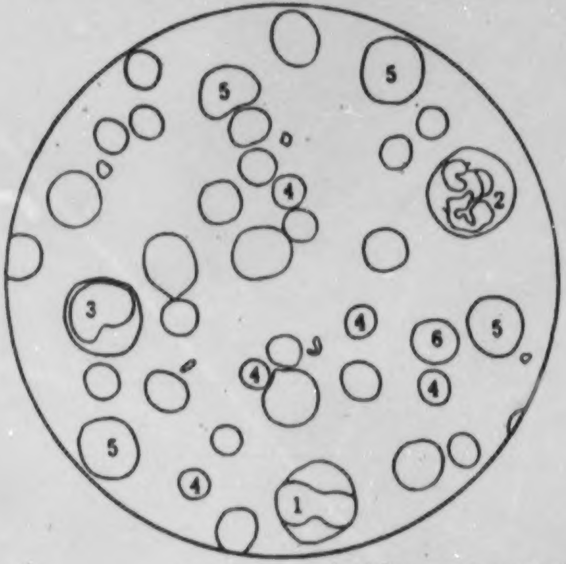
Sickle Cell Anemia. This is a chronic, hereditary, hemolytic anemia almost exclusively confined to Negroes. The red blood cells take on a crescent or sickle-shape in states of reduced oxygen tension. The sickle cell trait (a less severe form of the disease) occurs in approximately 10 percent of all Negroes but only one in forty has sickle cell anemia. These persons have a peculiar type of hemoglobin which has been demonstrated electrophoretically (hemoglobin "S").

Mediterranean Anemia (Cooley's Anemia or Thalassemia). This is a chronic, progressive, hereditary anemia commencing early in life and affecting persons of Mediterranean origin. It is characterized by the presence of thin, target type red cells in the peripheral blood. These cells have a decreased osmotic fragility. Immature red and white cells in the peripheral blood reflect the marrow activity.

Pernicious Anemia. This is a chronic macrocytic, normochromic anemia caused by a defect in the production of "intrinsic factor" by the stomach mucosa. There is megaloblastic hyperplasia of the bone marrow. The peripheral blood shows macrocytosis, poikilocytosis, polychromatophilia, granulocytopenia, and granulocytic hypersegmentation. Clinical features include achlorhydria and neurological disturbances.

Aplastic Anemia. This is a severe, usually normocytic anemia due to an interference with blood formation which may follow exposure to a variety of chemical or physical agents (benzene, arsenic, gold, radiation), or the disease may be of unknown origin.

CONGENITAL SPHEROCYTIC ANEMIA



1. Metamyelocyte
2. Segmented Neutrophil
3. Lymphocyte
4. Microspherocyte
5. Polychromatophilic Macrocyte
6. Normal Erythrocyte

The patient was a white male, 18 years old. Two months before admission he had an attack of precordial oppression, fatigue, shortness of breath and jaundice, from which he recovered after multiple transfusions at another activity.

The physical examination showed a well-developed, well-nourished male, of dark complexion with a slight yellowish tinge to the skin and sclerae. The spleen was just palpable.

Laboratory findings were: RBC 2,000,000; hemoglobin 6.2 gm%; WBC 13,200; myelocytes 1, metamyelocytes 2, band neutrophils 3, segmented neutrophils 50, lymphocytes 29, monocytes 11, eosinophils 4. Two normoblasts to 100 white blood cells. Reticulocytes were 42 percent. Platelets were normal. Erythrocytes showed marked polychromasia and anisocytosis with many microspherocytes, which appeared as small, round, deep staining red cells without central pallor. The fragility test showed hemolysis beginning at 0.78 percent sodium chloride and complete hemolysis at 0.40 percent sodium chloride. The control hemolysis began at 0.44 percent sodium chloride and was complete at 0.28 percent sodium chloride. Sternal puncture revealed a marked normoblastic hyperplasia and the presence of many microspherocytes.

At splenectomy, the organ was found to be four times normal size and contained a few infarcts. There was uneventful recovery. The platelets showed the usual post-splenectomy rise, numbering 1,200,000 on the tenth post-operative day, then gradually returned to normal. The microspherocytosis and increased fragility of red cells persisted after recovery but there was no anemia or reticulocytosis.

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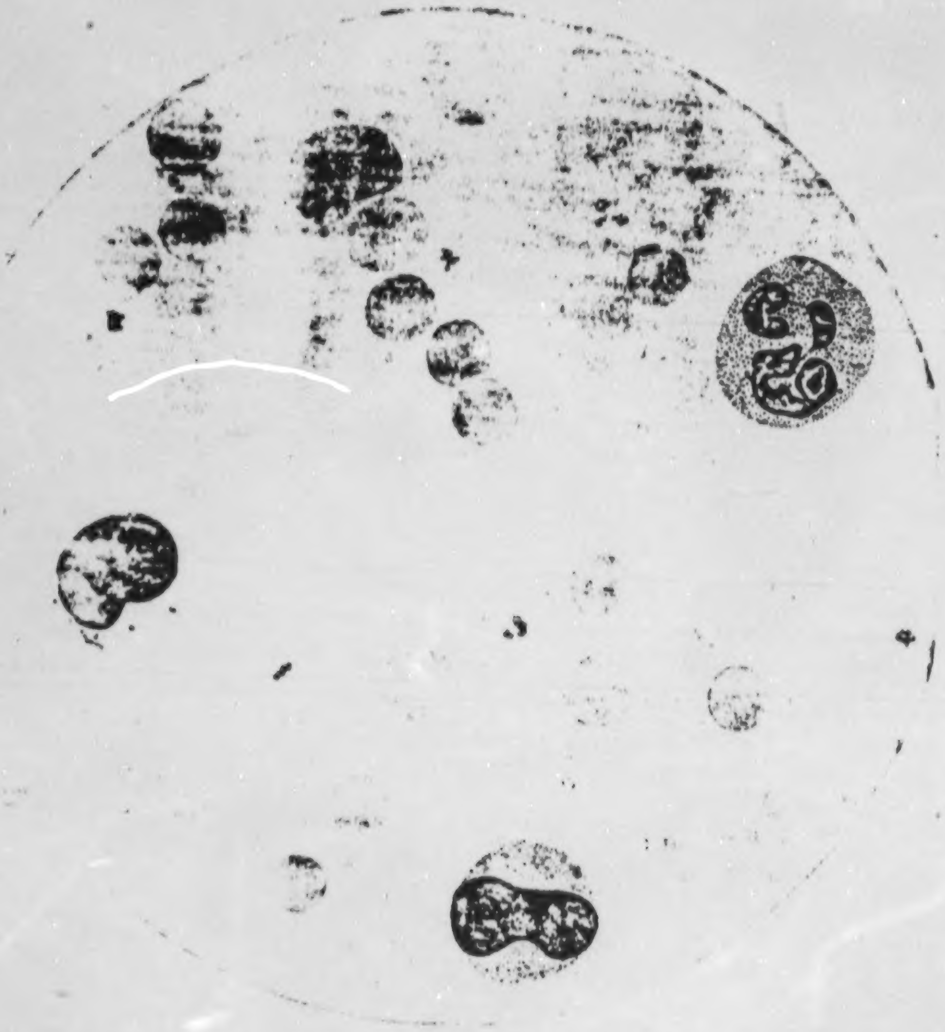
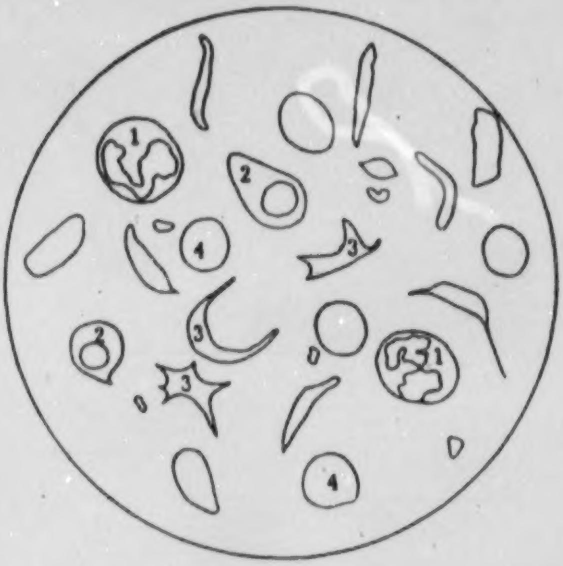


Congenital Spherocytic Anemia

SICKLE CELL ANEMIA

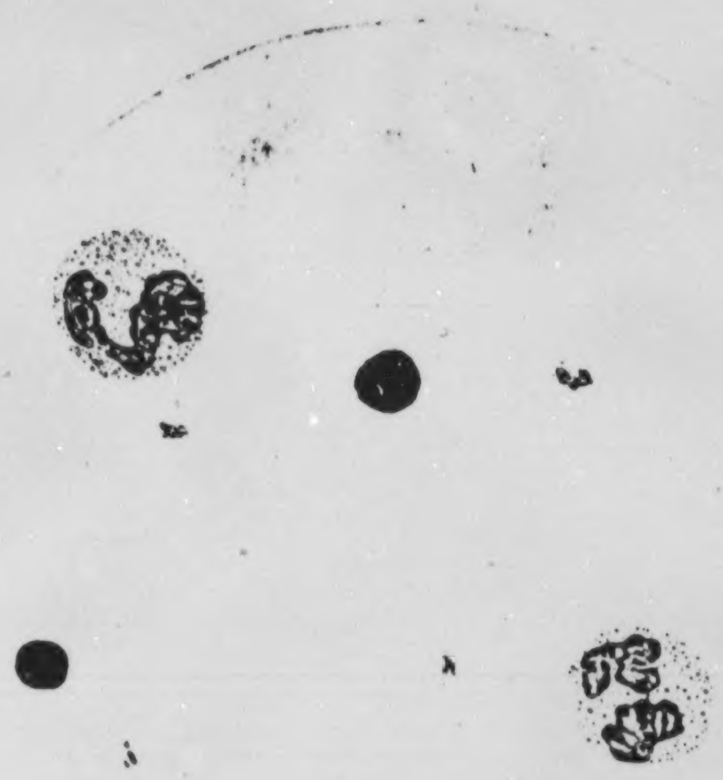


1. Segmented Neutrophil
2. Normoblast
3. Sickled Erythrocyte
4. Target Cell

The patient was a Negro male, age 23, admitted complaining of weakness, rheumatic pains of indefinite duration and a recent attack of acute pain in the splenic region.

Blood examination revealed RBC 2,100,000: hemoglobin 6.5 gm%; WBC 22,900: differential count, segmented neutrophils 77; lymphocytes 8; monocytes 15. Ten normoblasts were seen in counting 100 leukocytes. The red blood cells showed marked anisocytosis and poikilocytosis with a few bizarre, elongated and sickled forms. Reticulocyte count was 30 percent. Sealed wet preparation showed sickling in about 50 percent of the erythrocytes after 24 hours. Sternal bone marrow aspiration revealed a marked normoblastic hyperplasia and the same bizarre poikilocytosis that was evident in the peripheral blood smear. The sedimentation rate was 3 mm in 60 minutes. The fragility test showed increased resistance of the red cells to hypotonic salt solution with beginning hemolysis at 0.44 percent sodium chloride and incomplete hemolysis at 0.20 percent sodium chloride.

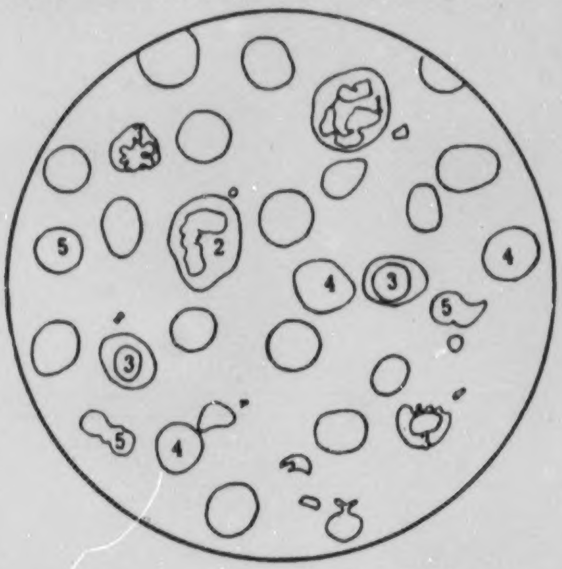
Sickle Cell Anemia



Sickle Cell Anemia

MEDITERRANEAN ANEMIA

(Cooley's Anemia; Thalassemia, major and minor)

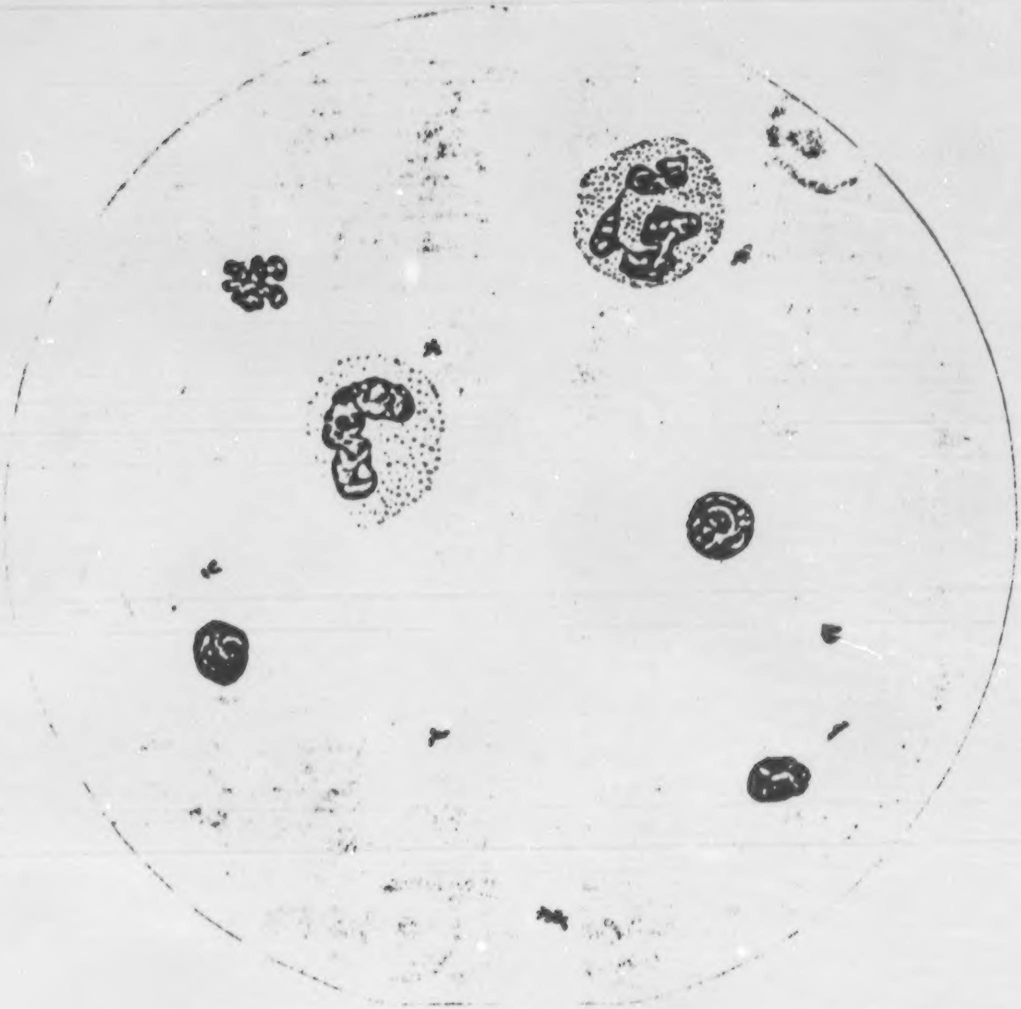


1. Segmented Neutrophil
2. Band Neutrophil
3. Normoblast
4. Target Cell
5. Poikilocyte

The patient was a white male, age 20, of Sicilian parentage, who complained of abdominal pain and weakness. Eight months prior to admission he had an acute attack of severe abdominal cramps, nausea, vomiting, fever with enlarged spleen and leukocytosis.

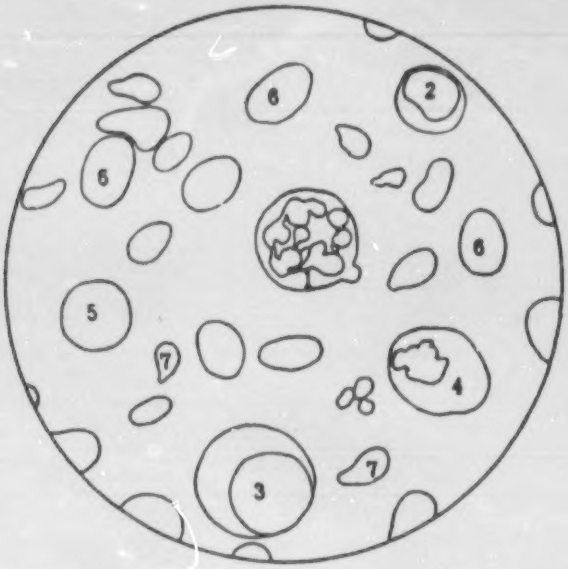
Physical examination revealed a well-developed man with a large spleen and slight jaundice. Blood studies revealed RBC 5,000,000; hemoglobin 11.5 gm%; WBC 16,200; band neutrophils 3, segmented neutrophils 77, lymphocytes 11, monocytes 7; eosinophils 1; basophils 1. The red blood cells showed many "target cells" and thin forms that had folded, also a few normoblasts. In the fragility test hemolysis began at 0.44 percent sodium chloride and was incomplete at 0.20 percent sodium chloride. The control began at 0.44 percent sodium chloride and was complete at 0.32 percent sodium chloride. Prothrombin time was 79 percent of normal. Icterus index was 15. Sternal puncture aspiration showed a very marked normoblastic hyperplasia and many target cells.

Mediterranean Anemia. (Cooley's Anemia; Thalassemia, Major and Minor)



Mediterranean Anemia. (Cooley's Anemia; Thalassemia, Major and Minor)

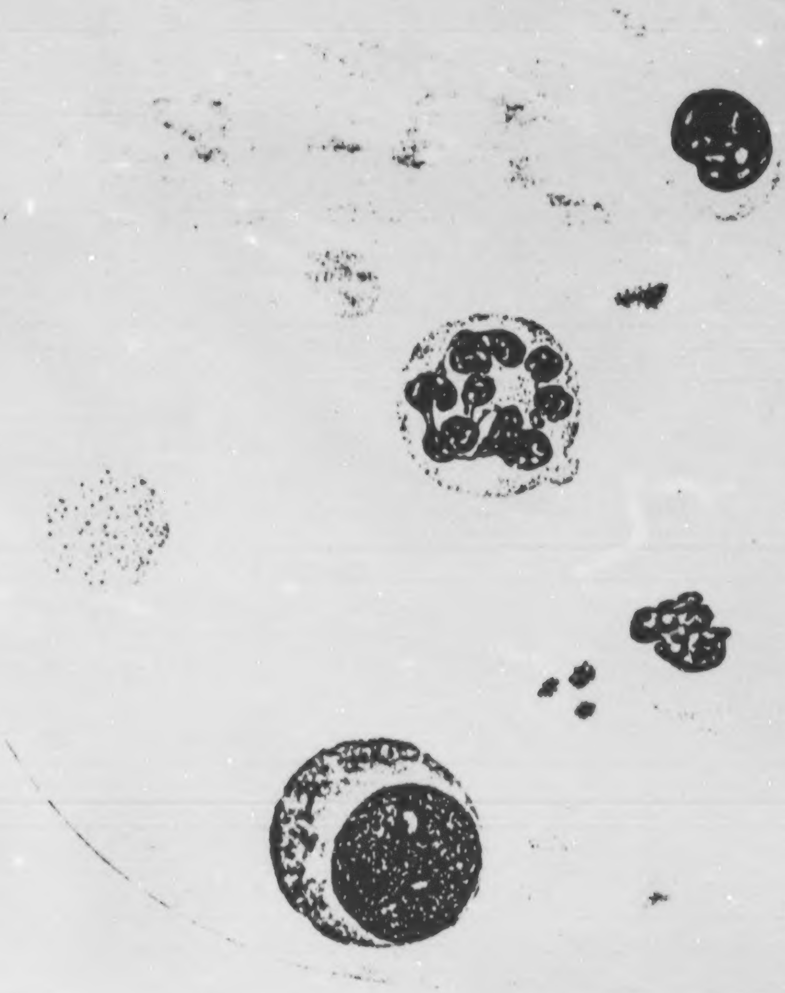
PERNICIOUS ANEMIA



1. Hypersegmented Neutrophil
2. Lymphocyte
3. Megaloblast
4. Macrocytic Normoblast
5. Stippled Erythrocyte
6. Macrocytic Erythrocyte
7. Poikilocyte

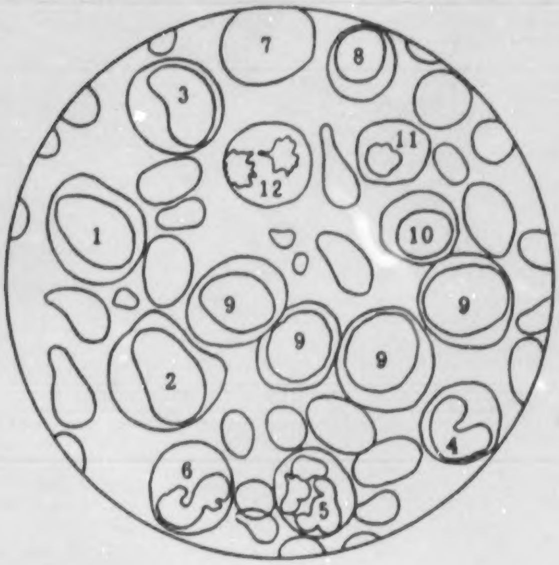
The patient was a white male, 60 years old, who complained of weakness, indigestion, sore tongue, and tingling with numbness of the hands and feet. He was well-nourished with a pale yellow skin and a smooth, red, glazed tongue. There was loss of vibratory sensation in the legs. The spleen was barely palpable. No free hydrochloric acid was present in the gastric juice after histamine stimulation. The blood findings were: RBC 1,400,000; hemoglobin, 5.5 gm%; volume of packed cells, 18.5 ml per 100 ml; WBC 4,400; myelocytes, 2; metamyelocytes 3; segmented neutrophils 56; lymphocytes 36; monocytes 2; eosinophils 1; platelet count 120,000. The majority of the neutrophils were enlarged with increased lobulations connected by distinct strands, the hypersegmented type or with a few lobes which were greatly elongated, the ribbon type. There were marked poikilocytosis, anisocytosis with macrocytosis, basophilic stippling, polychromasia and some large normoblasts with a rare megaloblast. The indices were: MCV, 132; MCH, 39; MCHC, 30; VI, 1.46; CI, 1.35. The sternal bone marrow aspiration showed a marked megaloblastic proliferation. A finding of more than 2 percent of megaloblasts in the bone marrow is characteristic of pernicious anemia.

Pernicious Anemia



Pernicious Anemia

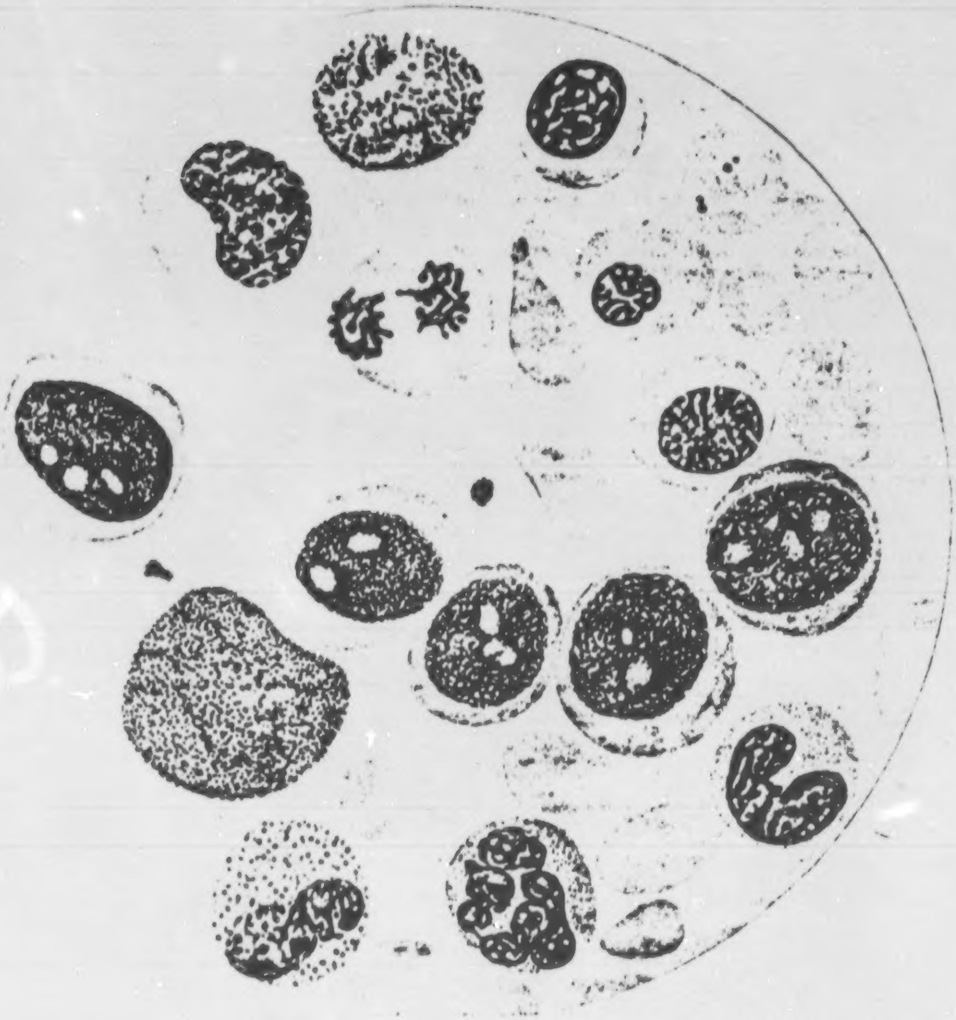
PERNICIOUS ANEMIA
(Bone Marrow)



1. Myeloblast
2. Promyelocyte
3. Neutrophilic Myelocyte
4. Metamyelocyte
5. Segmented Neutrophil
6. Eosinophil
7. Basophil
8. Lymphocyte
9. Megaloblast
10. Pronormoblast
11. Normoblast
12. Megaloblast, in mitosis

The bone marrow picture illustrated above is taken from the case of Pernicious Anemia described and illustrated on the preceding pages.

Pernicious Anemia. (Bone Marrow)



Pernicious Anemia. (Bone Marrow)

Chapter 5

LEUKOCYTE STUDIES

The cells of the leukocytic series are subject to quantitative and qualitative variations. Percentage-wise the quantitative variation of leukocytes is more apparent than the erythrocytes. Under stimulation the leukocyte response may be as great as 300–400 percent from normal, i.e., 20,000/cmm. Qualitative variations are also more apparent since variations may occur in either cell type or cell cytology or both. Some diseases stimulate the response of particular cell types and the predominant cell types furnish a diagnostic aid to the clinician, e.g., eosinophilia may indicate a parasitic infection or an allergy. Cytological structures may vary from the normal. These may indicate certain disease conditions such as infectious mononucleosis and Pelger-Huet disease. Other cytological variants occur quite often in malignancies such as leukemia and multiple myeloma. Because variations in numbers of cells are of such importance, detailed study of leukocytes must be made to determine their quantitative relationship to each other.

LEUKOCYTE DEVELOPMENT

All blood cells originate from primitive cells which have similar morphological characteristics. As primitive cells change from "blast" to more mature cell types, their nuclear and cytoplasmic characteristics change.

Mature cells are generally smaller than immature cells. The nuclear chromatin is more clumped and compact in the mature cells. Nucleoli are a sign of immaturity and are generally found in the more immature cells. The cytoplasm changes from the immature basophilic color to either a pink (granulocytes) or a blue to blue-gray (agranulocytes).

Definite and specific cytoplasmic granules develop in the cells of the granulocytic series as the cells mature. These granules are not to be found in the more immature cells of this series. Nonspecific granules (azurophilic) will be found in the earlier stages of the granulocytic series and often throughout the young and the mature stages of the agranulocytic series.

GRANULOCYTE MATURATION

The granulocytic series refers to the development of the granular leukocytes. These cells generally originate in the bone marrow, but under some circumstances may be produced in other organs such as the spleen and the liver. As the granulocyte matures, it becomes characterized by the development of metachromatic nonspecific cytoplasmic granules, and later, more specific granules. These specific type granules are the basis for differentiation of the granulocytes. The stages in the normal maturation of the granulocytes are:

Myeloblast
Promyelocyte
Myelocyte (neutrophilic, eosinophilic, and basophilic)
Metamyelocyte (neutrophilic, eosinophilic, and basophilic)
Band cell (neutrophilic, eosinophilic, and basophilic)
Segmented cell (neutrophilic, eosinophilic, and basophilic)

Myeloblast

Myeloblast. These cells vary in diameter from 10–20 microns. The nucleus is round and stains predominantly reddish purple. It is separated from the cytoplasm by a definite nuclear membrane. The interlaced chromatin strands are delicate, well-defined and evenly stained. Two or more nucleoli are usually demonstrable.

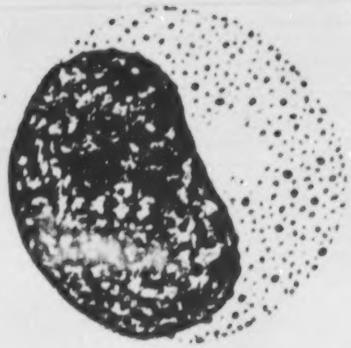
There is a moderate amount of nongranular cytoplasm, which forms a thin rim around the nucleus.

Promyelocyte

Promyelocyte. These cells generally have a greater amount of cytoplasma than myelocytes. The nucleoli, if visible, are not as well defined as in the myeloblasts and the chromatin strands tend to be coarser and less evenly stained. Another principal difference lies in the presence of varying numbers and sizes of dark nonspecific granules which stain from red to purplish blue (azurophilic). A cell ceases to be a promyelocyte and becomes a myelocyte when specific granules are formed to the degree that one can tell by the color size and shape of the granules that the cell is a basophil, eosinophil, or neutrophil.

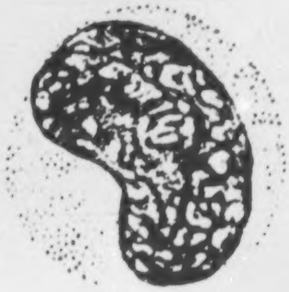
Myelocyte. These are more mature cells of the granulocytic series than the promyelocyte. A few *specific* granules may be formed in the promyelocyte stage. In the myelocyte stage they are definite and so numerous that they frequently obscure nuclear details. While promyelocytes may sometimes be distinguished as being neutrophilic, eosinophilic, or basophilic, the differentiation is generally considered as first occurring in the myelocytic stage.

DEVELOPMENT OF THE NEUTROPHILIC GROUP



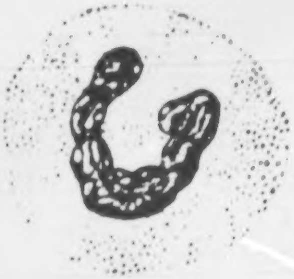
Neutrophilic Myelocyte

Neutrophilic Myelocyte. The first sign of neutrophilic myelocyte differentiation or "dawn of neutrophilia" takes place in the form of a small, relatively light island of ill-defined, dark pinkish granules which develops within the bluish cytoplasm and among the dark, nonspecific metachromatic granules of the promyelocyte. As the myelocyte ages the dark granules become less prominent and the specific neutrophilic granules predominate. The nucleus is round, nucleoli are usually indistinct or absent, and the chromatin strands are unevenly stained and thickened. Neutrophilic myelocytes are usually smaller than promyelocytes and have relatively larger amounts of cytoplasm.



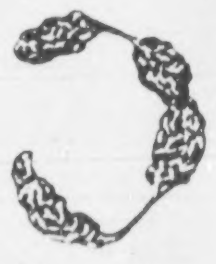
Neutrophilic Metamyelocyte

Neutrophilic Metamyelocyte. Neutrophilic metamyelocytes have a slightly indented nucleus and small pinkish-blue granules. As a class, they are slightly smaller than myelocytes and have a relatively smaller nucleus and less well-defined chromatin structures. Neutrophilic metamyelocytes are rarely seen in the peripheral blood in normal individuals, but are often found in conditions in which there is myelocytic hyperplasia.



Neutrophilic Band

Neutrophilic Band. As the neutrophilic metamyelocytes mature, the nuclear indentation becomes more marked. In the band form the nuclear indentation is slightly more than half the width of the hypothetical round nucleus. The opposite edges of the nucleus become approximately parallel for an appreciable distance. Neutrophilic bands are slightly smaller than metamyelocytes. There is usually a dark pyknotic mass at each pole of the nucleus where the lobe is destined to be. The specific cytoplasmic granules of band neutrophils are small, evenly distributed, and stain various shades of pink.



Neutrophilic Segmented Cell

Neutrophilic Segmented Cell. The mature neutrophils are approximately twice the size of erythrocytes. These cells differ from neutrophilic bands in that the nucleus has definite lobes separated by a very narrow filament or strand, rather than a wide band. The cytoplasm in an ideal Wright's stained preparation is light pink and the small, numerous and evenly distributed neutrophilic granules have a light pink color.

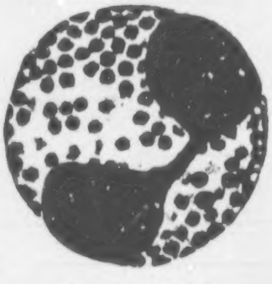
The transition between the various stages of neutrophilic cells is gradual. Many borderline cells are difficult to distinguish. Cells should be placed in the more mature category when there is doubt. For example, if a question arises as to whether a cell is a metamyelocyte or a band, it should be tallied in the band column. Likewise, questionable bands should be called segmented.

The lobes of segmented neutrophils often touch one another or are superimposed which makes it impossible to see the connecting filament. This is particularly true of thick films or where the cells are more spherical and the structures less well defined. If the margin of a given lobe can be traced as a definite line from one side of the isthmus across to the other side, it is assumed that the lobes are superimposed and that the cell is segmented even though the filament is not visible.

To attempt to classify granulocytes into their various developmental stages is useless unless the smears are very thin and properly stained. A good microscope must be available and the technician must be well trained in the details of morphological hematology. Otherwise it may be more practical to classify granulocytes as neutrophils, eosinophils or basophils without attempting to determine their stage of maturation. Unless the more refined (Schilling) differential counts are critically made and the reports are reliable for clinical evaluation, they should not be attempted. The differential count, however, is an exceedingly valuable procedure.

Development of the Eosinophilic Group

Cells of the eosinophil group are characterized by relatively large, spherical cytoplasmic granules which have a particular affinity for the eosin stain. The earliest eosinophils (promyelocyte) have a few dark spherical granules with reddish tints which develop among the dark, nonspecific granules. As the eosinophilic cells pass through their various developmental stages, these granules become less purplish-red and more reddish-orange. The dark blue, nonspecific granules, characteristic of the promyelocyte and the early myelocyte stages, disappear. Because the percentage of eosinophils is usually low in bone marrow or peripheral blood smears, no useful clinical purpose is served by routinely separating the eosinophils into their various myelocyte, metamyelocyte, band and segmented categories. On the other hand, in situations such as eosinophilic leukemia in which the eosinophils are greatly increased, an analysis of the incidence of the various stages would be useful in diagnosis.



Mature Eosinophil

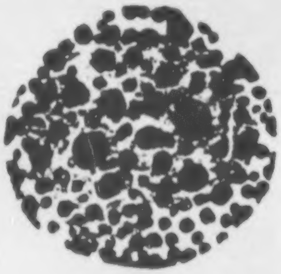
Mature Eosinophil. The eosinophils seen in normal peripheral blood smears are about the size of neutrophils and usually have a band or a two-lobed nucleus. The granules are spherical, uniform in size, and are usually evenly distributed throughout the cytoplasm, but rarely overlie the nucleus. In good stains the granules take a bright reddish-orange stain. On focusing up and down one can bring out highlights on individual granules which make them appear as little spheres. Often this spherical shape will help identify the cell when the stain is unsatisfactory. Eosinophils can be readily identified without the use of strains because the granules are uniform and so distinct, round, and relatively large

Frequently it is difficult to distinguish eosinophils from neutrophils when the granules of the neutrophils are prominent and the stain dark. In case of doubt, the questionable cell should be called a neutrophil. Guesswork can be avoided by moving to the thin part of the smear where there is brighter illumination. If close attention is paid to the size, uniformity and shape of the granules rather than to the color alone, these cells can be more easily differentiated.

Development of the Basophilic Group

These cells have round, indented, band or lobulated nuclei which are classified according to the shape of their nuclei, as basophilic myelocytes, metamyelocytes, bands and segmented forms. These cells are so few in peripheral blood and bone marrow that there is no clinical advantage in placing the cells in separate age catgories.

Basophils with round nuclei (basophilic myelocytes) may be indistinguishable from promyelocytes for both have dark granules. Also, there is an absence of eosinophilic or neutrophilic granules and a round nucleus in both. As a rule basophilic myelocytes are smaller, have less cytoplasm and a more mature nucleus. In case of doubt it is recommened that the cell be called a progranulocyte rather than a basophilic myelocyte.



Mature Basophil

Mature Basophil. The granules range in color from dark blue to black. These granules are usually visible above as well as lateral to the nucleus. The granules are unevenly distributed and vary in number, size, shape and color. They are often adjacent to, or are surrounded by, a relatively unstained area.

Neutrophilic granules may be darkly stained or have prominent so-called "toxic granules" which superficially resemble basophilic granules. The size of neutrophil granules is usually sufficient to make a differential diagnosis. In doubtful cases, the cell is interpreted as a neutrophil.

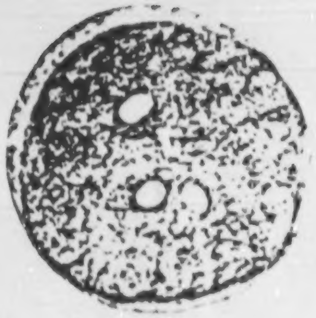
AGRANULOCYTE MATURATION

The agranulocytic series is comprised of leukocytes devoid of specific granulation. These cells generally originate in the lymphatic system, but like the granulocytic series, they may be produced elsewhere in the body. This series includes the lymphocytic, monocytic, and plasmocytic group.

Development of the Lymphocytic Group

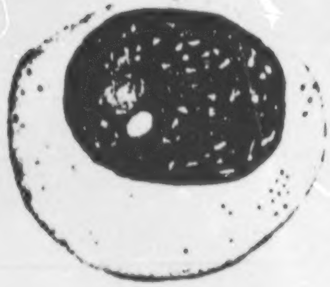
The lymphocytic series refers to the development of the lymphocytes. These cells arise mainly from the reticular tissue of the lymph nodes and lymphoid tissue from which they derive their name. The stages in the development of the lymphocytic series are:

Lymphoblast
Prolymphocyte
Lymphocyte



Lymphoblast

Lymphoblast. These cells are similar to the other blasts. The are round or oval, from 10–20 microns wide, and have a round or oval reddish-purple nucleus. The nuclear chromatin is fine and well distributed but perhaps more coarse than in myeloblasts. Chromatin is condensed at the edges to form a definite nuclear membrane. One or two nucleoli may be present. The cytoplasm is bluish and nongranular and forms a thin rim around the nucleus.



Prolymphocyte

Prolymphocyte. These cells are 16–25 microns wide and are round or oval in shape. The nucleus of the cell is also round or oval and red-purple. The nuclear chromatin is coarse and slightly clumped. Nucleoli may be present. There is an abundant amount of light blue cytoplasm around the nucleus. There also may be a few red-purple (azurophilic) globules present.

Lymphocyte

Lymphocyte. These are the mature cells of the lymphocytic series and they vary greatly in size. Small lymphocytes are 9–12 microns in diameter, while large lymphocytes may be 20–30 microns wide. However, size does not determine the age of these cells. The cells are easily distorted and, consequently, often appear in irregular shapes in stained preparations. The nuclear chromatin is markedly condensed to form large, discrete, almost solid clumps, with thickening of the nuclear membrane. Nucleoli are absent. The cytoplasm of the cells is blue. Often tiny colorless globules appear in the cytoplasm which resemble granules. Care should be taken not to confuse these globules with specific granules as the lymphocyte is agranular.

Lymphocytes are the most undifferentiated of all blood cells normally found in the peripheral blood. The nucleus does not become segmented and specific granules do not develop. Their cytoplasm does not develop past the blue stage. It is difficult to distinguish young from old cells, except on the basis of the chromatin pattern.

Development of the Monocytic Group

The monocytic series refers to the stages of development of the monocyte. Monocytes may be formed from reticuloendothelial cells wherever such cells occur in the body. One of the most important sites of origin is the spleen. The reticular cells may give rise to monoblasts which divide actively and mature to form monocytes. The stages in their development are:

Monoblast
Promonocyte
Monocyte

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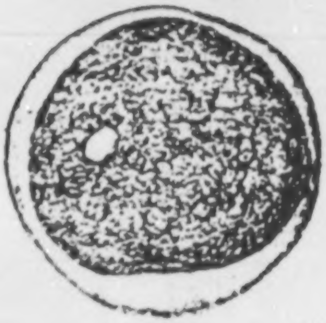
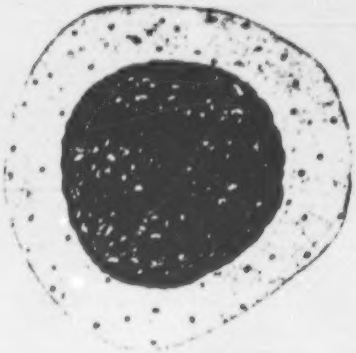


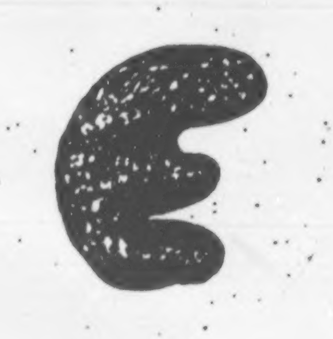
Monoblast

Monoblast. These cells are 10–20 microns wide. The nucleus is round or oval and red-purple in color. The nuclear chromatin is fine and well-distributed. A nucleolus is present. The deep, clear blue cytoplasm is a thin rim around the nucleus. There are no granules present.



Promonocyte

Promonocyte. These cells are 14–20 microns wide. The nucleus is irregularly-shaped and light red-blue. The nuclear chromatin is fine and spongy. There may be a nucleolus present. The cytoplasm is gray-blue with fine red-purple (azurophilic) globules.



Monocyte

Monocyte. Monocytes are larger than neutrophils in the thin portions of a smear where the cells tend to flatten out. In thicker areas of a smear there is less difference in comparative cell size. The size varies between 15–25 microns. Monocytes have slow ameoboid movement and often become dried with single or multiple pseudopods.

The nuclei of the monocytes are usually round or kidney-shaped, but may be deeply indented or have two or more lobes connected by narrow bands. One of the most distinctive and diagnostic features of the monocyte is the presence of superimposed lobes, giving the nucleus the appearance of brainlike convolutions. Heavy lines marking the edges of the folds and grooves are features which are not seen in other cells. Another feature of the nucleus, which is of value in diagnosis, is the tendency for the nuclear chromatin to be loose with light spaces in between the chromatin strands,

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giving a coarse linear pattern in contrast to the lymphocyte which has clumped chromatin. Nucleoli are absent.

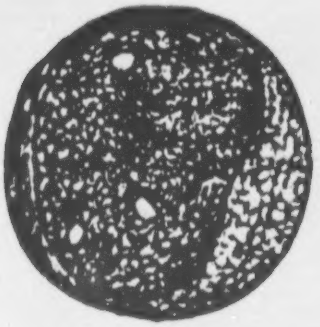
There is a large amount of cytoplasm in relation to the nucleus. The cytoplasm of the monocyte is dull gray-blue while the cytoplasm of the neutrophils in the adjacent fields is definitely lighter and is pink rather than gray-blue. The nonspecific granules of the monocyte are usually fine and evenly distributed, giving to the cell a dull, opaque or ground-glass appearance. In addition to the background of evenly distributed nonspecic granules, there may be a few unevenly distributed larger azurophilic granules. Vacuoles are often demonstrable in the cytoplasm. Erythrocytes, nuclei of other cells, cell fragments and pigment may be phagocytized.

Promonocytes and monoblasts, except in monocytic leukemias, are not identified as such in bone marrow or peripheral blood. These cells may have pseudopods, indented nuclei with folds and grooves, vacuoles, and they have the ability to phagocytize. They possess the nuclear characteristics of early cells.

Development of the Plasmocytic Group

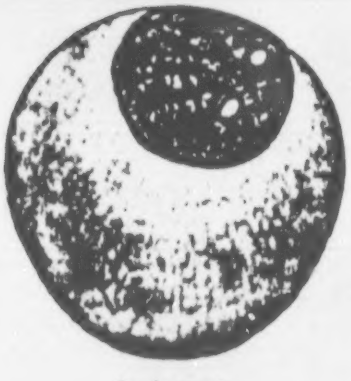
Plasmocytes constitute approximately one percent of the nucleated white [illegible] normal bone marrow where they tend to be grouped in small islands around blood vessels. They are not seen in the smears of peripheral blood of normal persons, but may be present in small numbers in chronic infections, granulomatous and allergic diseases, and plasma cell myeloma. The stages of development of the cells in the plasmocytic series are as follows:

Plasmoblast
Proplasmocyte
Plasmocyte



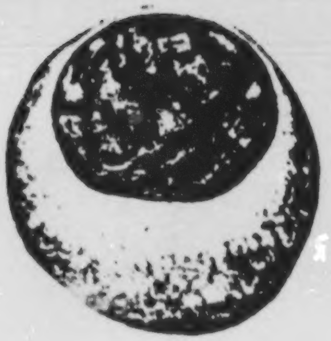
Plasmoblast

Plasmoblast. Plasmoblasts resemble other "blast" cells. The nucleus is large, round or oval, and contains within the fine, reticulated chromatin meshwork, one or two nucleoli. The cytoplasm is relatively scanty and stains dark blue. No granules or vacuoles are found in the cytoplasm. Plasmoblasts and proplasmocytes are distinguished from the mature plasmocyte by their nuclear characteristics. The size of the cell and the intensity of the blue color are not reliable criteria of age. Plasmoblasts are not recognizable except in malignancies of the plasmocytic type, in which cases cells are often abnormal.



Proplasmocyte

Proplasmocyte. The nuclei of these cells show more clumping and the chromatin is not as finely reticulated as in plasmoblasts. Nucleoli are present. The nucleus may be eccentrically located in the cytoplasm. The cytoplasm is pale blue, with pale reddish-blue vacuolation. A pale perinuclear zone, or "halo" is usually present.



Plasmocyte

Plasmocyte. The mature plasmocyte varies greatly in size. The plasmocytes usually have an oval shape with relatively smooth cytoplasmic margins. However, like the lymphocyte, the plasmocyte is easily traumatized and may have frayed or nebulous margins and pointed or filamentous cytoplasmic projections. Plasmocytes have a large amount of cytoplasm and a small and usually eccentric nucleus.

The nuclei of plasmocytes are round and contain coarse, lumpy chromatin. The so-called "cart wheel" nucleus characterized by tendency of the chromatin to attach to the rim of the nuclear membrane, like the spokes of a wheel, is observed in fixed tissue preparations and probably is an artifact due to shrinkage. In air-dried blood and bone marrow smears, the "cart-wheel" effect is not usually present although, in some texts, it is stated to be a characteristic feature of the mature plasmocyte.

The cytoplasm adjacent to the nucleus is lightly stained in contrast to the periphery of the cell which has a high saturation of red and blue dyes. In some cells the dark cytoplasm has a greenish or larkspur-blue color. The cytoplasm contains multiple small and relatively unstained globules embedded in a bluish-red filamentous matrix. It is the presence of these tapiocalike globules in the dark surrounding medium which gives the plasmocyte its characteristic mottled and foamy appearance and its brilliant translucency. In occasional cells the globules may be quite prominent and take a red or bluish-red stain. Such globules are called Russell or fuchsin bodies, or eosinophilic globules. Vacuoles of various sizes are frequently demonstrable.

DIFFERENTIATION BETWEEN MONOCYTES, MYELOCYTES, AND LARGE LYMPHOCYTES

Monocytes are the cells of the peripheral blood which are most difficult to identify and to differentiate from other cells. The nucleus may be round or only slightly indented, the linear chromatin pattern ill-defined, and the distinctive blunt pseudopods and digestive vacuoles may be lacking. They are frequently mistaken for immature neutrophils because of prominent granular and lobulated nuclei. They are often mistaken for large lymphocytes, for their cytoplasm is blue, while the nonspecific granules may be indistinct.

The two most characteristic features of the monocyte, and the most helpful in recognition, when other differential features are absent, are the brainlike convolutions of the nucleus and the dull gray-blue color of the cytoplasm.

The color of the cytoplasm must not be compared with a textbook picture of hypothetical ideal cells, but rather with known neutrophils in the same or adjacent fields. Brainlike convolutions are not seen in myelocytes and metamyelocytes. The cytoplasm of a neutrophil is relatively light and reddish in comparison with the cytoplasm of the monocyte which is relatively dark, more bluish and opaque. Neutrophils practically never have blunt pseudopods.

In order to distinguish monocytes from large lymphocytes, the nuclear structure, the character of the cytoplasm, and the shape of the cells are most useful. The character of the nucleus of a lymphocyte tends to be clumped, rather than strandlike, or linear. There is a greater tendency for the nuclear chromatin to be condensed at the periphery in the lymphocyte. Brainlike convolutions are not present in the lymphocyte. Both monocytes and large lymphocytes may have azurophilic granules. In the monocytes granulation tends to be evenly distributed throughout the cytoplasm. Granulation of the lymphocytes tends to be aggregated near the nuclear indentation. Lymphocytes and monocytes may have scalloped, irregular or hollyleaf shapes, but lymphocytes are more likely to have pointed projections. Lymphocytes are more easily indented by adjacent erythrocytes than are monocytes.

In thick or poorly stained smears and in many pathological bloods, it is impossible to identify positively all cells and to differentiate all monocytes from myelocytes, metamyelocytes, or large lymphocytes. Such questionable cells are identified "by the company they keep." In situations where there is no predominance of one cell or a mixed association without clearly defined transitional forms, one should report such questionable cells as atypical and describe their morphological characteristics.

EOSINOPHIL COUNT

(Pilot)

Principle (Ref: 20, 23)

A sample of blood is diluted with a solution which selectively stains the eosinophils and eliminates all other leukocytes and erythrocytes from view. Following mixing, the specimen is introduced into the counting chamber and the number of eosinophils in a known volume of blood is counted.

APPARATUS

Finger puncture material
Thoma white blood cell diluting pipettes
Hemacytometers with coverglasses

REAGENT

Pilot's solution (22)

TECHNIQUE

1. Draw capillary (or venous blood) to the 1.0 mark in each of two white cell diluting pipettes.
2. Draw Pilot's solution to the 11 mark of each pipette.
3. Mix by gently shaking the pipettes for 30 seconds. Prolonged and harsh shaking will tend to cause rupturing of the eosinophils.
4. Expel the cell-free liquid from the capillary portion of the pipettes.
5. Using one pipette, charge both chambers of a hemacytometer and with the other pipette charge both chambers of the second hemacytometer.
6. Allow both hemacytometers to stand for 15 minutes to permit staining of the eosinophils. To prevent evaporation, the hemacytometers may be placed on a damp towel and covered with Petri dish covers.
7. Under low power magnification, count the red-stained eosinophils in the entire ruled area (9 sq mm) on each of the four chambers (a total area of 36 sq mm).

CALCULATION

The actual count can be calculated by substituting in the following formula:

$$\frac{\text{Number of eosinophils} \times \text{dilution (10)} \times \text{depth (10)}}{\text{Area (36)}} = \text{eosinophils/cmm}$$

NORMAL

150–300 eosinophils/cmm

DISCUSSION

1. Fuschs-Rosenthal or Levy hemacytometer is preferable to a standard counting chamber since its greater volume (4.0 x 4.0 x 0.2 mm) allows for counting of more cells thereby reducing the statistical error. Counting two of these chambers is equivalent in accuracy to seven standard chamber counts.
2. In eosinopenia, it is necessary to set up more chambers to provide an optimum number of cells to be counted.
3. The eosin-acetone diluting fluids are unsatisfactory and should not be used.
4. Estimation of eosinophils on a stained blood smear is too inaccurate for use because of poor cellular distribution.
5. The propylene glycol in Pilot's solution renders the erythrocytes invisible and the sodium carbonate causes lysis of all the leukocytes except the eosinophils. The phloxine stains the eosinophils.
6. In the Thorn test an eosinophil count must be made prior to the initiation of the test proper. This establishes the patient's total eosinophil count, to which the response of the adrenal cortex to ACTH can be judged. ACTH is then injected and at an interval of 4 hours, another eosinophil count is made. The interpretation of this test is as follows:

   Normal—approximately a 50 percent drop in eosinophils
   Cushing's disease (hyperadrenalism) —0–30 eosinophils/cmm
   Addison's disease (hypoadrenalism) no change in eosinophil count

SOURCES OF ERROR

1. Use of old Pilot's solution.
2. Debris in diluent.

3. Squeezing finger to obtain blood.
4. Failure to draw blood to, but not beyond, the "1.0" mark deposits a thin film of cells even though the column is immediately brought down to the mark; this could produce an elevated count.
5. Inaccurate pipetting of diluting fluid. Fluid should be pipetted in a continuous motion to, but never beyond, the "11" mark. The washing backward and forward of blood and diluent in the pipette in an attempt to hit exactly the "11" mark overly dilutes the specimen.
6. To slow manipulation following withdrawal of the specimen thus [illegible] some of the blood specimen to coagulate.
7. Failure to mix blood specimen and diluent thoroughly.
8. Overly harsh or prolonged shaking, causing rupture of eosinophils and lower count.
9. Improper charging of the counting chamber.
10. Too short a standing period, or allowing to stand uncovered.
11. Uneven distribution of cells in counting chamber.
12. Inaccuracy in counting cells.

CEREBROSPINAL FLUID CELL COUNTS

Principle (Ref: 13, 20, 33)

Cerebrospinal fluid is normally water-clear and cell free. If leukocytes are present, they may be counted and examined directly in a hemacytometer. They may be diluted with a fluid which hemolyzes any red cells present. They may be examined on a stained smear.

APPARATUS

Thoma WBC diluting pipette
Hemacytometer with coverslip
Microscope and lamp
Rubber tubing with mouthpiece

REAGENT

Cerebrospinal fluid diluting fluid (6)
Hayem's solution

TECHNIQUE

Note: Cell counts on spinal fluid should be made within 30 minutes after withdrawal of the specimen to avoid cellular disintegration.

1. With a pipette, introduce an undiluted drop of well-mixed spinal fluid from the specimen into one counting chamber of hemacytometer.
2. Examine the entire ruled area for the presence of cellular elements. If both leukocytes and erythrocytes are seen, note the condition of the red cells (fresh or crenated).
    a. If the total cell content is low (less than 500), count all the cells and calculate cellular content according to the calculations for Step 2a below.
    b. If the total cell content is high (over 500) and difficult to count, the specimen can be diluted (1/10) in a white cell dilution pipette with Hayem's solution and the total cells counted. Calculations are in accordance with those for Step 2b below.
3. Draw the spinal fluid diluting fluid to the 1.0 mark of the white blood cell dilution pipette.
4. Draw a well-mixed specimen of spinal fluid to the 11 mark of the pipette.
5. Shake the pipette for two minutes to mix the specimen.
6. Discard the fluid in the capillary portion of the pipette.
7. Charge the counting chamber and allow the cells to settle for five minutes.
8. Under low power magnification, count all cells in the entire ruled area (9 square mm). Calculations are below.
9. Switch to high power and perform a rough differential count.

CALCULATIONS

For Step 2a:

$$\frac{\text{Cells counted} \times \text{depth } (10) \times \text{dilution } (1)}{\text{area } (9)} = \text{cells/cmm}$$

For Step 2b:

$$\frac{\text{Cells counted} \times \text{depth } (10) \times \text{dilution } (10)}{\text{area } (9)} = \text{cells/cmm}$$

For Step 8:

$$\frac{\text{Cells counted} \times \text{depth } (10) \times \text{dilution } (10/9)}{\text{area } (9)} = \text{cells/cmm}$$

NORMALS

0–10 lymphocytes/cmm

DISCUSSION

1. If more than 100 leukocytes per cmm are present, centrifuge the undiluted specimen, make a smear and stain with modified Wright's stain. Perform a routine differential count and also estimate the ratio of erythrocytes to leukocytes.

Note: It may be necessary to use egg albumin or cell-free serum to make the sediment adhere to the slide.

2. Normally the spinal fluid is water-clear. It may be turbid if cell count is 500 or more cells per cmm. If there is frank blood with spontaneous clotting, the indications are those of a bloody tap. Xanthochromia develops after subarachnoid hemorrhage has been present for a few hours and is due to disintegration of blood pigments. Xanthochromia may also develop from tumors, abscesses and inflammation.
3. Cell counts above 10 are considered to be evidence of intracranial disease. The predominant cell in most viral infections, lues and tuberculous meningitis is the lymphocyte. Pyogenic infections due to meningococcus, pneumococcus, etc., usually result in a predominance of the neutrophil. Cerebral and extra dural abscesses, as well as subdural hemorrhages produce a neutrophilic response although no bacteria can be demonstrated.
4. Biochemical, bacteriological, virological, serological, and hematological examinations are all necessary to reflect the true condition of the cerebrospinal fluid. The current laboratory standing operating procedures should give guidance to the efficient method to accomplish all the necessary examinations.

STUDY OF STAINED BLOOD SMEARS AND THE DIFFERENTIAL LEUKOCYTE COUNT

Principle (Ref: 13, 16, 17, 20, 33)

The stained blood smear permits the study of the appearance and the identification of the different kinds of leukocytes, and the appearance of erythrocytes and thrombocytes(blood platelets).

APPARATUS

Microscope and lamp
Differential counter

TECHNIQUE

1. Examine the stained blood smear under low power magnification in order to get an appraisal of the general distribution of cells and staining quality of the smear.
2. Next examine the smear under high dry magnification to detect any abnormal or unusual cells. If such cells are present, switch to oil immersion magnification for their identification.
3. Select a thin, well-stained area under high power magnification and switch to the oil immersion objective.
4. Study the red cells and report any evidence of rouleaux formation or signs of immaturity (polychromatophilia, normoblasts, nuclear remnants, etc.).

Always report the erythrocyte morphology with reference to size, shape, and staining characteristics. If nucleated red blood cells are found, *report their number per 100 leukocytes* counted.

5. Observe the thrombocytes in several oil immersion fields to obtain a rough estimation of their numbers (normal, increased, or decreased). Normal is an average of 4–6 per oil immersion field. Note any abnormality in morphology (giant platelets, etc.). If the thrombocytes appear to be significantly decreased, a thrombocyte count and/or a clot retraction test may be indicated.
6. Identify and count 100 consecutive leukocytes and record each cell type separately on the differential counter. Begin at the thin end of the smear and count the white cells observed as the slide is moved in a *vertical* direction. When near the edges of the smear, move the slide horizontally for a distance of about two fields, then proceed vertically back across the smear. Continue this "snake-like" movement until 100 leukocytes have been counted and classified.
7. The number of each type of leukocyte is expressed as a percent of the total number of white cells counted. Absolute values may be calculated by multiplying the percent value by the total leukocytic count.

NORMALS (Adults)

	Percent	Absolute Values/cmm
Neutrophilic metamyelocytes	0-1	0-100
Neutrophilic bands	3-5	150-500
Segmented neutrophils	55-65	2750-6500
Eosinophils	2-4	100-400
Basophils	0-1	0-100
Lymphocytes	20-35	1000-3500
Monocytes	2-6	100-600

DISCUSSION

1. In blood smears, prepared by the slide method, leukocytes are not distributed evenly throughout the smear. The monocytes and granular leukocytes tend to aggregate near the edges of the smear while the lymphocytes remain in the central portion. In doing [illegible] ferential count on a preparation made by the slide method, the cells at the extreme edges of the smear should not be evaluated.

2. In routine laboratory work, the counting of 100 consecutive leukocytes on a stained blood smear is usually sufficient. However, if the differential leukocyte count is abnormal, an additional 100 white cells should be counted and the percent calculated.

3. All abnormal white cells (e.g., immature, hypersegmented, toxic, atypical lymphocytes, etc.) should be classified or described and reported in percent separately. Cells that are ruptured, fragmented, or degenerated are *not* included in the differential count, but should be noted separately and reported as the number seen per 100 leukocytes.

4. In view of the gradual transition in the form of the metamyelocyte to the band and then to the segmented granulocyte, exact classification is sometimes difficult. In such cases, classify the cell according to the more mature form.

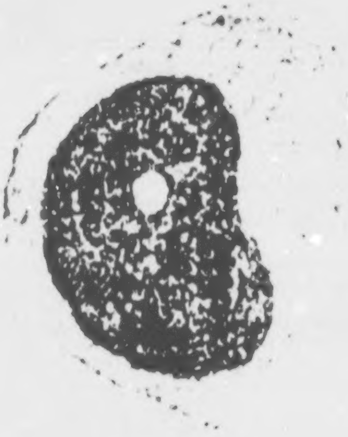
THROMBOCYTES

Because the thrombocytes are concerned with coagulation, the maturation series and enumeration of the thrombocytes are considered in the present chapter. Microscopic evaluation of the megakarocytes in the bone marrow may be of importance in hemostasis, especially when considering the purpuras.

The general pattern of thrombocyte maturation is slightly different from that of leukocyte maturation. The cells of the megakaryocytic series tend to grow larger as they mature until there is cytoplasmic fragmentation (or breaking off) to form the cytoplasmic thrombocytes seen in the peripheral blood.

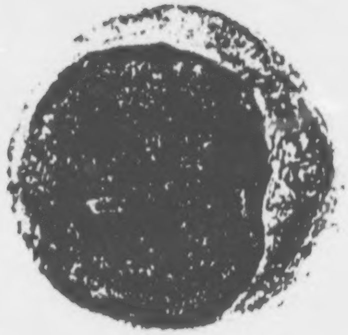
Azurophilic granulation begins to appear in the second stage of development and continues until it almost obscures the nuclear lobes. The nucleus develops from a fine single lobe to multiple ill-defined lobes. The stages in the normal maturation of the megakaryocytic series are:

Megakaryoblast
Promegakaryocyte
Megakaryocyte
Metamegakaryocyte
Thrombocyte



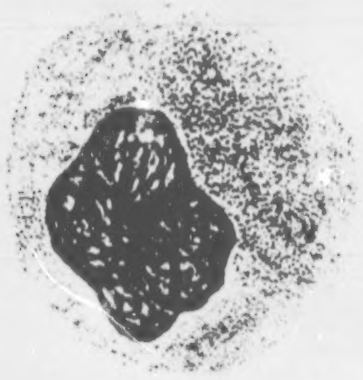
Megakaryoblast

Megakaryoblast. These cells are usually fixed tissue cells and, as such, are rarely seen in routine bone marrow preparations. They range from 20–30 microns in diameter, and are irregular in shape. There is a nucleolus in the nucleus. The scanty cytoplasm is blue and lacks granulation.



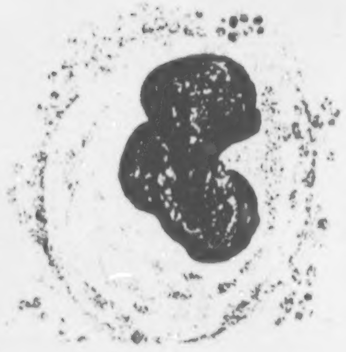
Promegakaryocyte

Promegakaryocyte. These cells are larger than the preceding cells and quite often have an indented or double nucleus. Azurophilic granules may be present. The cytoplasm usually shows some buff coloration.



Megakaryocyte

Megakaryocytes and Metamegakaryocytes. These cells range from 50–100 microns in diameter. They have multilobes that are usually ill-defined. The cytoplasm has many azurophilic granules but does not show evidence of fragmentation of thrombocytes. Metamegakaryocytes are giant cells and much like the megakaryocyte but show evidence of fragmentation or sloughing of thrombocytes from the cytoplasmic margins.



Metamegakaryocyte

Thrombocytes. These cytoplasmic fragments are small (1–3 microns in diameter). The cytoplasm appears light blue while the centrally located azurophilic granules appear purplish red. There is no nucleus in these cells.

Thrombocyte

Note: Normally, megakaryocytes and metamegakaryocytes do not appear in the peripheral blood, however, they may be seen in some leukemias. In bone marrow preparations, there should be little difficulty in recognizing cells as being of this series because of their enormous size.

THROMBOCYTE COUNTS

DIRECT THROMBOCYTE COUNT

Principle (Ref: 1, 20, 32, 33)

A sample of blood is diluted in an isotonic anticoagulant. The anticoagulant employed contains a stain and serves the dual purpose of being both a fixing diluent and a staining medium. After mixing the suspension is introduced into the counting chamber and the number of thrombocytes in a known volume is counted.

APPARATUS

Finger puncture material
Pipette, Thoma red blood cell diluting
Counting chamber

REAGENT

Rees-Ecker diluting fluid (26)

TECHNIQUE

1. Fill a red cell diluting pipette with freshly filtered Rees-Ecker solution and expel it, leaving a film of the solution inside the pipette.
2. Draw capillary blood to the 0.5 mark in the red cell pipette.
3. Wipe the excess blood from the outside of the pipette and dilute to the 101 mark with Rees-Ecker diluting fluid.
4. Shake the pipette for several minutes. Discharge a few drops from the pipette. Then charge both chambers of a hemacytometer.
5. Allow the thrombocytes to settle for 15 minutes. To prevent drying, the counting chamber may be placed on a damp towel and covered with a Petri dish cover.
6. Under high power magnification, count all of the thrombocytes seen in the center 1 sq mm area of each of the two charged chambers of the hemacytometer. With proper adjustment of the light and continual fine focusing, the thrombocytes are seen as blue, highly refractile bodies, which may be round, oval, or comma-shaped. They vary in size from 1–5 microns.

CALCULATION OF THE DIRECT THROMBOCYTE COUNT

$$\frac{\text{Total thrombocytes counted} \times \text{dilution (200)} \times \text{depth (10)}}{\text{Area (2)}} \quad \text{EQUALS thromb}$$

EXAMPLE:

Total thrombocytes counted:	250
Dilution factor:	200
Depth factor:	10
Area factor:	2

$$\frac{250 \times 200 \times 10}{2} = 250{,}000 \text{ thrombocytes cmm}$$

NORMAL VALUES

200,000–400,000 thrombocytes cmm

Note: This range is generally acceptable but it must be realized that there is a great variance in normal values which differ with each technique, laboratory, and technician.

DISCUSSION

1. No one method for the enumeration of thrombocytes is satisfactory in every respect. In experienced hands the direct procedure is more accurate than the indirect method and venous blood samples are more representative and preferable to those obtained by capillary puncture. Direct procedures, however, require greater skill and experience for proper performance and interpretation. The error in a single count has been estimated to be about 10 percent, and this margin of error can be reduced by multiple counts.
2. A major disadvantage of the direct thrombocyte count is that the platelets must be counted under high power rather than oil immersion magnification as is used in the indirect method. Because of the extremely small size of blood platelets, it is a common error to confuse them with yeasts, debris, and precipitated stain.
3. Due to the inherent errors in this procedure, it is recommended that a thrombocyte count be performed on a normal person as a control.

SOURCES OF ERROR

1. Improperly cleaned counting chambers, coverslips, and pipettes.
2. Squeezing the finger following the puncture to obtain blood.
3. Contaminated, old, or unfiltered diluting fluid. Extraneous matter in the diluting fluid or in any of the glassware may be interpreted as platelets by even the most experienced technician. Therefore, the following precautions are recommended:
   a. Boil counting chambers and coverslips for 2 hours in distilled water to which a small amount of sodium bicarbonate has been added.
   b. Pipettes must be very carefully cleaned.
   c. The diluting fluid must be frequently filtered or centrifuged and stored at 2°–4°C.
4. Other sources of error as listed under red and white blood cell counts also apply, including pipetting errors. These procedures should be consulted.
5. More accuracy may be obtained through use of the phase contrast microscope.

INDIRECT THROMBOCYTE COUNT (Fonio)

Principle (Ref: 20, 33)

Thrombocytes are counted indirectly by determining their ratio to red blood cells in a stained blood smear. Using this ratio and the total red cell count, the number of platelets per cmm may be calculated.

APPARATUS

Finger puncture material
Pipette, Thoma red blood cell diluting
Counting chamber
Glass slide

REAGENTS

Magnesium sulfate, 14 percent, sterile (19)
Hayem's diluting fluid (13)
Wright's stain and buffer (modified) (37) (38)

TECHNIQUE

1. Clean and dry the finger tip and place a large drop of 14 percent magnesium sulfate on the site to be punctured.
2. Puncture through this drop and allow the blood to well up and mix with the magnesium sulfate. *Do not squeeze* the finger to obtain blood nor mix the blood and solution with the lancet.
3. Touch a clean glass slide to this mixture and prepare and stain a thin blood smear.
4. Cut a 5-mm hole in a circular piece of paper the size of a 5-cent piece. Place the disc in the ocular of the microscope to reduce the size of the field.
5. Focus on the stained blood smear under oil immersion magnification. Locate the thin portion of the smear. Count 200 red blood cells in each of five different fields. Count and record the number of thrombocytes per 1,000 red blood cells. Thrombocytes appear as blue cytoplasmic bodies, 1–5 microns in diameter, containing numerous red purple (azurophilic) granules.
6. Perform a red blood cell count on the patient. The technique for the RBC count is described in chapter 4.

CALCULATIONS

$$\frac{\text{Red blood cell count/cmm} \times \text{thrombocytes counted}}{\text{Red blood cells counted on stained blood smear}} \quad \text{EQUALS thrombocytes/cmm}$$

EXAMPLE:

Red blood cell count:	4,500,000/cmm
Red cells counted on smear:	1,000
Platelets counted on smear:	80

$$\frac{4{,}500{,}000 \times 80}{1{,}000} = 360{,}000 \text{ thrombocytes/cmm}$$

NORMAL VALUES

200,000–400,000 thrombocytes/cmm

DISCUSSION

1. This procedure is less accurate than the direct method, but it requires less skill and experience to perform.
2. The major disadvantages of the in-

direct method of counting thrombocytes are:

a. The red blood count, upon which the thrombocyte calculation is based, is subject to error.

b. Platelets may not be distributed evenly throughout the smear.

c. The thromocytes will be obscured in thick blood smears.

3. The magnesium sulfate prevents the agglutination and disintegration of the thrombocytes.

SOURCES OF ERROR

1. Squeezing finger.
2. Delay in running test. Additional time will allow for marginal migration of the erythrocytes.
3. Debris in the magnesium sulfate or RBC dilution fluid.
4. Precipitated Wright's stain.

CLOT RETRACTION

(MacFarlane's Method—Modified)

Principle (Ref: 18, 31, 32)

This test evaluates the functions of thrombocytes. A measured amount of blood is allowed to clot around a wire hook in a graduated centrifuge tube. After coagulation, the clot is removed and volume of serum measured. The volume of serum is reported as a percentage of the total possible serum obtained by a hematocrit determination. This test measures the degree of contraction of the clot in a specified period of time.

APPARATUS

Venipuncture material
Centrifuge tube, graduated
Cork with wire hook attached
Hematocrit tube
Test tube with Heller-Paul anticoagulant

TECHNIQUE

1. Draw 6 ml of venous blood. Place 4 ml in a clean dry graduated centrifuge tube. Place the remainder of the blood into the anticoagulant tube and mix well.
2. Place the cork holding the wire hook in the mouth of the centrifuge tube so that the wire extends well beneath the surface of the blood, (see fig. 1).
3. Place the centrifuge tube in a 37°C. water bath until the clot forms. Allow the tube to remain in the water bath for an additional hour.
4. Carefully free the clot from the sides of the centrifuge tube. Allow the clot to drain well on the side of the tube, then remove it from the serum.
5. Centrifuge the serum-cell mixture at 2,000 rpm for 15 minutes.
6. Obtain the packed cell volume and the total volume from the graduations on the centrifuge tube. Subtract the cell volume from the total volume to determine the volume of expressed serum.
7. Fill the Wintrobe hematocrit tube from the well-mixed anticoagulated specimen and centrifuge at 3,000 rpm for 30 minutes. Read the percent of *plasma*.
8. Calculate the percent serum expressed according to the formula given in the calculations.

CALCULATIONS

$$\frac{\text{Volume of expressed serum} \times 100}{\text{Percent plasma (hematocrit)} \times \text{total blood volume in centrifuge tube}} \text{ EQUALS percent serum expressed}$$

EXAMPLE:

Volume of expressed serum: 2.0 ml
Hematocrit: 55.0% plasma, 45% total cells
Total blood volume in centrifuge tube: 4.0 ml

$$\frac{2.0 \times 100}{0.55 \times 4.0} = 91\% \text{ serum expressed}$$

NORMAL VALUES

62–94% serum expressed (Mean 78%)

DISCUSSION

1. This procedure is basically that of MacFarlane but includes the corrections for

hematocrit and for red cells not trapped in the clot. The percentage of plasma (derived from the hematocrit) serves as an index of the total fluid which could be expressed from the clot. These refinements increase the accuracy of the procedure.

2. When the rate of clotting is unusually slow or imperfect, the method is unreliable, since incomplete coagulation renders the clot nonadherent and easily displaced.
3. Positive correlation of platelet numbers and clot retraction is such that a decrease in clot retraction (expressed serum) is a more reliable index of thrombocytopenia than the platelet count. A normal degree of clot retraction is strong evidence that the thrombocyte count is normal.
4. The clot retraction is normal in hemophilia since there is a normal number of platelets. However, the onset of contraction is often delayed in blood samples from hemophiliac patients.
5. A screening method of clot retraction may be done by placing the blood sample, maintaining sterile technique, in a dry test tube which is then incubated. The time that retraction begins (30–60 minutes) and the degree of retraction (poor, partial, complete) in 24 hours is observed.

SOURCES OF ERROR

1. Dirty glassware
2. Maceration of clot during extraction.
3. Errors associated with performance of hematocrit.
4. Presence of residual anticoagulants in centrifuge tube.
5. Presence of plasmin (fibrinolysin) in specimen.
6. Inaccuracy in reading.

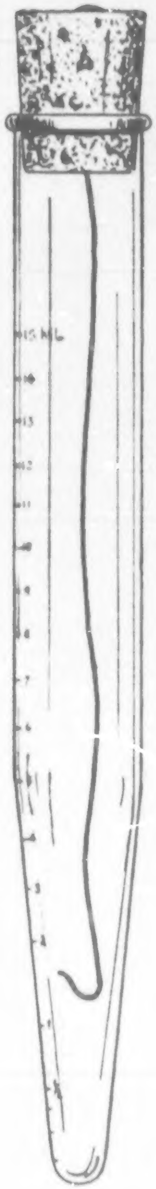


Figure 1. Centrifuge Tube With Wire Hook Used in Clot Retraction Test.

SERUM PROTHROMBIN CONSUMPTION TEST

Principle (Ref: 20, 30)

Prothrombin is partially used up in the normal clotting process. The amount of prothrombin remaining in serum indicates the adequacy of the thromboplastin complex. The time required for a clot to form when optimal amounts of thromboplastin, fibrinogen, calcium and serum are mixed is the serum prothrombin consumption time.

APPARATUS

- Venipuncture material
- Wassermann tubes
- Medicine dropper
- Pipettes, serological, blow-out type (5.0 ml, 1.0 ml, 0.2 ml, 0.1 ml)
- Kahn tubes
- Stop watch
- Nichrome wire loop, No. 22

REAGENTS

- Simplastin (29)
- Fibrinogen (11)

TECHNIQUE

1. Draw 5 ml of blood by a *nontraumatic* venipuncture.
2. Place the blood in a test tube and allow it to clot at room temperature.
3. Place the clotted blood in a water bath at 37°C. for 1 hour.
4. Centrifuge the specimen at 3,000 rpm for 3 minutes and transfer the serum to another test tube.
5. Mix 2 ml of Simplastin and 1 ml of fibrinogen solution in a clean dry Wassermann tube.
6. Pipette 0.2 ml of this mixture into each of three Kahn tubes and place the tubes in a 37°C. water bath for 5 minutes.
7. Place the serum from step 4, in a water bath at 37°C. for 5 minutes.
8. Transfer 0.1 ml of serum to the first of the three Kahn tubes by quickly blowing out the contents of the pipette and starting the stop watch simultaneously.
9. Determine the time for the clot to form as in the plasma prothrombin test.
10. Repeat the procedure commencing with step 8 for tubes number two and three.
11. The three determinations should agree as follows:

Serum-Prothrombin consumption time	*Variation*
over 30 seconds	2.0 seconds
20-30 seconds	1.0 seconds
12-20 seconds	0.5 seconds

NORMAL VALUES

Longer than 30 seconds:	Normal
20-30 seconds:	Doubtful
Less than 20 seconds:	Abnormal

DISCUSSION

1. Patients having decreases or defects in in the thromboplastin complex (thrombocytopenia, hemophilia, deficiency in plasma thromboplastin antecedent or component), will not use up normal amounts of prothrombin in the clotting process. The excess of residual prothrombin in the serum will result in a serum prothrombin time of less than 20 seconds.
2. Administration of heparin will serve to shorten the prothrombin consumption time as well as prolong the "Lee-White" clotting time. Dicumarol, on the other hand, will have no effect upon the serum prothrombin time, even while prolonging the plasma prothrombin time. This indicates that dicumarol has no influence upon the thromboplastin complex.

SOURCES OF ERROR

1. Failure to refrigerate the serum if it is not to be tested immediately.
2. Failure to mix Simplastin and fibrinogen just prior to use.
3. Improper temperature. All reagents must be warmed to 37°C. for 5 minutes just prior to use. This incubation time is critical.
4. Improper preparation of Simplastin or use of outdated Simplastin.
5. The presence of circulating anticoagulants (anti-thromboplastin).
6. Traumatic venipuncture. This encourages an increase of tissue thromboplastin.

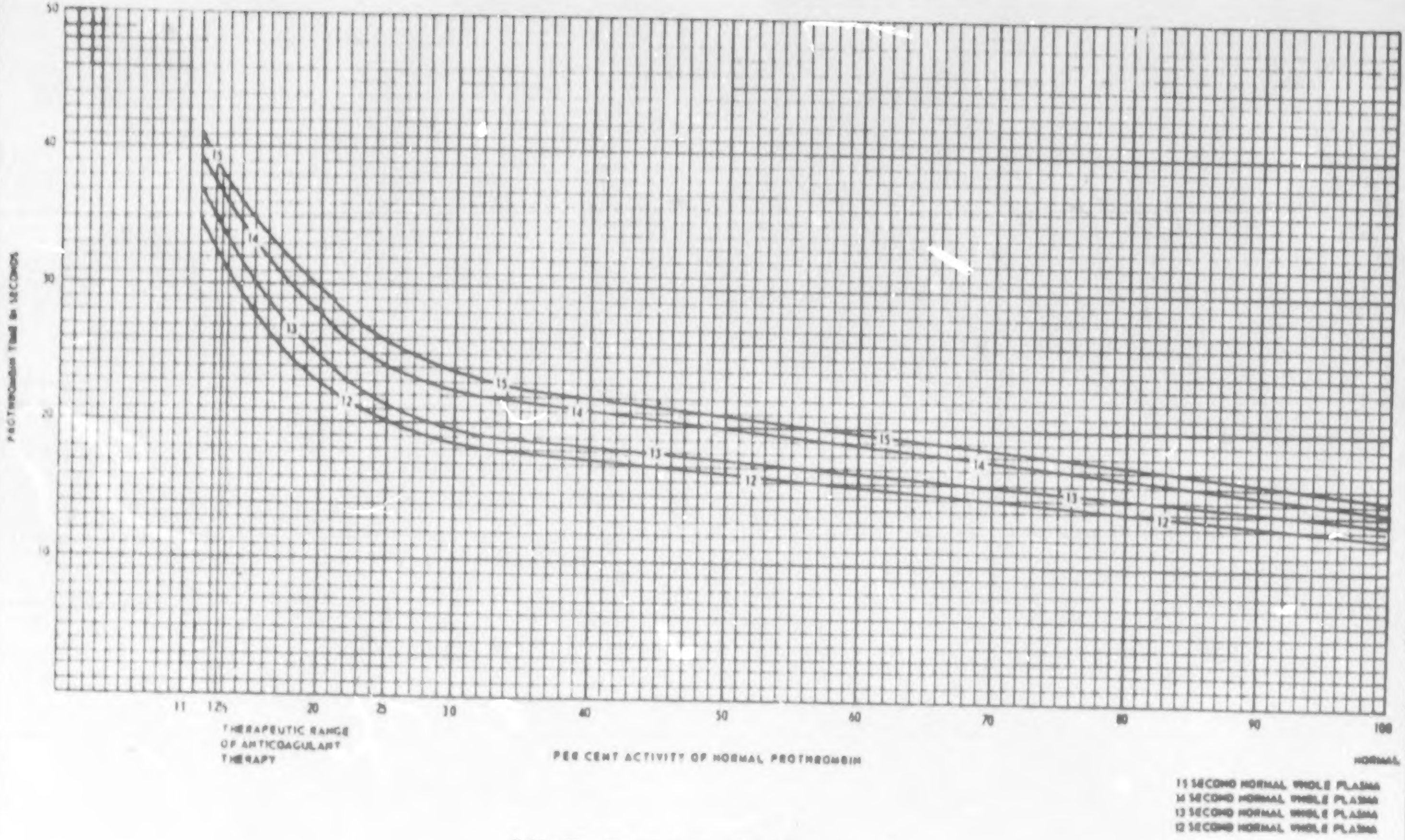


Table III. Typical Prothrombin Activity Curves

PROTHROMBIN TIME

(Quick's One-Stage Method Using Simplastin)

Principle (Ref: 4, 20, 25, 32, 33)

When optimal amounts of thromboplastin, calcium, and oxalated plasma are mixed under carefully controlled conditions, fibrin strands will normally form within a matter of seconds. The interval during which this reaction occurs is the prothrombin time. This test measures the overall prothrombin activity of whole plasma.

APPARATUS

Venipuncture material
Wassermann tubes
Graduated centrifuge tube containing 0.5 ml of 0.1 M sodium oxalate
Pipettes, serological, blow-out type (0.2 ml, 0.1 ml)
Kahn tubes
Stop watch
Nichrome wire loop, No. 22

REAGENTS

Sodium oxalate, 0.1 M (34)
Simplastin (28)
Diagnostic plasma (8)

TECHNIQUE

1. Draw 5 ml of blood by a nontraumatic venipuncture. Apply the tourniquet *just prior* to performing the venipuncture.
2. Immediately add *exactly* 4.5 ml of blood to 0.5 ml of sodium oxalate, 0.1 M, contained in a graduated centrifuge tube.
3. Thoroughly mix the blood and anticoagulant by gently inverting the tube several times.
4. Centrifuge the blood specimen (within 30 minutes) at 1,700 rpm for 10 minutes.
5. Draw off the clear plasma (free of cells) into a clean Wassermann tube and place the tube in a 37°C. water bath for 5 to 10 minutes.

Note: Refrigerate the plasma if the test cannot be performed immediately.

6. Pipette 0.2 ml of Simplastin (thromboplastin extract containing calcium chloride) into each of three Kahn tubes and place in a wate 37°C. for 3 minutes.
7. Transfer 0.1 ml of plasma into the first of the three Kahn tubes by quickly blowing out the contents of the pipette and starting the stop watch simultaneously.
8. Immediately mix the plasma and Simplastin by gently shaking the tube in the water bath.
9. Insert a No. 22 nichrome wire loop into the tube and move it across the bottom of the tube in sweeping motions about two times per second.

Note: The "tilt-tube" method may be used, if preferred. This involves periodically tilting and observing for clot formation.

10. The instant the clot adheres to the loop, stop the watch and note the time. This is the prothrombin time in seconds.
11. Repeat the procedure commencing with step 7 for tubes numbers 2 and 3.
12. The three determinations should agree as follows:

Prothrombin time	*Variation*
12–20 seconds	0.5 second
20–30 seconds	1.0 second
Over 30 seconds	2.0 seconds

13. Run a normal control with diagnostic plasma to evaluate the Simplastin.
14. Determine the degree of prothrombin activity by comparison of the results of the patient's plasma and the normal control by reference to table III.

NORMAL VALUES

12–15 seconds

DISCUSSION

1. When thromboplasin preparations other than Simplastin are used, the directions of the manufacturer must be followed exactly.

2. The activities of different preparations vary and, therefore, normal controls should be run to evaluate the activity of each preparation.

3. The prothrombin time of blood from a normal person must be determined each day. This serves as a normal control against which all tests performed that day may be compared. This control value must be reported with each patient's value. A prepared normal control of plasma is now available commercially. Abnormal results in the control indicate defective Simplastin.

4. The prothrombin activity may be reported in percentage as well as in seconds by applying the patient's prothrombin time (in seconds) to a prothrombin activity curve. Curves for different normal controls are supplied by the manufacturer. If the plasma dilution curve is to be prepared, whole normal plasma must be diluted with prothrombin free plasma. Saline should never be used in making the dilutions. The percentage activity should never be calculated by dividing the normal time by the patient's prothrombin time.

5. The prothrombin activity of a patient's plasma has important diagnostic and prognostic significance in diseases of the liver in vitamin K deficiency, and in the use of dicumarol as a therapeutic anticoagulant.

6. Prolongation of the one-stage prothrombin time does not measure prothrombin deficiency alone but rather indicates some failure of conversion of prothrombin to thrombin. This process involves prothrombin, proaccelerin (labile factor) and proconvertin (stable factor). Varying reductions in any one, or combination of these factors, will prolong the one-stage prothrombin time.

SOURCES OF ERROR

1. Improper timing. The stop watch must be started the instant the plasma comes into contact with the Simplastin and it must be stopped immediately when the clot forms.

2. Improper preparation of the Simplastin.

3. Traumatic venipuncture.

4. Improper temperature. The test must be run at the temperature of 37°C. Exposure of plasma in the 37°C. water bath must not be prolonged. All equipment (pipettes, etc.) should be at a temperature of 37°C.

5. Improper application of the tourniquet. It must not be prolonged.

6. Waiting too long to run the test. The test must be accomplished within 3 hours after collection of the blood specimen. If the test is not run immediately, the plasma and Simplastin must be refrigerated.

7. Delay in adding blood to anticoagulant.

8. Improper time and speed of centrifugation.

9. Improper proportions of blood and anticoagulant.

10. Inadequate cleansing of glassware. After each use, the glassware must be scrupulously cleaned with soft soap and a test tube brush and thoroughly rinsed. Synthetic detergents *should not* be used. Unscratched glassware reserved solely for prothrombin determinations should be utilized.

11. Use of outdated Simplastin.

12. Spillage of water. Water accidently introduced into the reagent tubes invalidates the test results.

FIBRINOGEN SCREENING TEST

(Warner-Chilcott)

Principle (Ref: 22)

In this test, the controls are diagnostic plasma diluted to 125 mg% of fibrinogen or lower. These controls are clotted with Simplastin and the test plasma is treated in the same manner. The approximate range of the fibrinogen is calculated by observation of the quality of the clot.

APPARATUS

Venipuncture material
Wassermann tubes
Centrifuge tube, graduated, containing 0.5 ml of 0.1 M sodium oxalate
Pipettes, serological, blow-out type, 0.2 ml and 0.1 ml
Kahn tubes
Stop watch
Nichrome wire loop, No. 22

REAGENTS

Simplastin (29)
Diagnostic plasma (8)

TECHNIQUE

Test Sample.

1. Draw a blood sample to obtain plasma as described in the procedure for a prothrombin time determination.
2. Add 0.1 ml of plasma to 0.7 ml of 0.85 percent sodium chloride. Mix thoroughly.
3. Blow 0.1 ml of the diluted plasma into 0.2 ml of Simplastin which has been placed in a 10 x 75 mm test tube.
4. Allow the mixture to stand for 5 minutes at room temperature.
5. At the end of the 5-minute-incubation period, observe clot formation by gently tilting the tube to a horizontal position. Do not shake the tube or tilt it repeatedly. Observation of the clot may be enhanced by noting the reflection of the clot in the concave surface of a microscope mirror.

Control Sample.

1. Reconstitute one vial of diagnostic plasma as specified on the container label as for prothrombin time.
2. Prepare the following dilutions in 0.85 percent sodium chloride.

To diagnostic plasma	*Add sodium chloride (0.85 percent)*	*Gives fibrinogen concentration (mg per 100 ml)*
0.1 ml	1.5 ml	150
0.1 ml	2.3 ml	100
0.1 ml	3.1 ml	75
0.1 ml	4.7 ml	50

3. Blow 0.1 ml of each of the diluted plasma samples into 0.2 ml amounts of Simplastin which have been transferred to 10 x 75 mm test tubes.
4. Allow the mixtures to stand for 5 minutes at room temperature.
5. At the end of the 5-minute incubation period, observe clot formation in the same manner as with the test sample. The control samples should be run simultaneously with the patient's sample.
6. Compare known controls with patient's test.

INTERPRETATION

1. A solid or large sliding clot—sufficient fibrinogen (over 150 mg per 100 ml).
2. Moderate-sized sliding clot with some liquid—borderline fibrinogen concentration (75 to 125 mg per 100 ml).
3. Small clot to fibrin strands—low fibrinogen (under 75 mg per 100 ml).
4. No visible sign of fibrin formation—very low fibrinogen (less than 30 mg per 100 ml).

Note: To determine the size and characteristics of the clot, compare the test to the control tubes.

DISCUSSION

1. The 5-minute incubation period allows possible low prothrombin levels in the tested specimen to form sufficient thrombin to convert fibrinogen into fibrin. With prothrombin levels as low

as 1 percent of normal, valid fibrinogen levels may be found.

2. Because fibrinogen deficiencies in whole plasma do not become evident by clotting procedures until the fibrinogen level is down close to 10 percent of normal, this test is performed on the plasma dilutions of 12.5 percent or below. The reading obtained allows accurate approximation of the fibrinogen level in the original whole plasma.
3. If there are no visible signs of fibrin formation, high titers of plasmin (fibrinolysin) should be excluded before low concentrations of fibrinogen are recorded.

SOURCES OF ERROR

1. Improperly prepared or use of outdated Simplastin.
2. Excessive agitation of tubes.
3. Residual anticoagulants incorporated in test.
4. Presence of circulating anticoagulants and plasmin.

HEMORRHAGIC DISEASES

Diseases manifesting themselves as bleeding dysfunctions may be grouped into several categories. The categories into which hemorrhagic diseases may be conveniently divided are based upon the area of deficiency, i.e., intravascular, a combination of both intravascular and vascular, or circulating anticoagulants and abnormal protein. This grouping aids greatly in the laboratory diagnosis of these diseases as well as in their clinical treatment.

HEMORRHAGIC DISORDERS ASSOCIATED WITH INTRAVASCULAR DEFICIENCIES

The intravascular hemorrhagic diseases may be due to deficiencies in thromboplastin activity, the prothrombin complex, fibrinogen, or circulating anticoagulants. Each of these deficiencies is discussed in the sections which follow.

Deficiency in Thromboplastin Activity

This group of hemorrhagic disorders is characterized primarily by reduced thromboplastin formation. These disorders may be caused by a deficiency in the platelet thromboplastin factor, antihemophilic factor, plasma thromboplastin component, or plasma thromboplastin antecedent.

True hemophilia is among the most commonly recognized forms of the hemorrhagic disorders caused by deficiency in thromboplastin activity. This disease manifests itself by a lifelong tendency to hemorrhage, and occurs mainly in males. The less severe forms may be overlooked since they demonstrate little, if any, tendency to bleeding. This disease is a mendelian sex-linked familial trait which follows the well-known pattern of being transmitted to male offspring by carrier mothers who do not manifest a bleeding tendency. Genetically, it is a recessive trait transmitted on the X-chromosome. Rarely, hemophilia occurs in females and, when it does, it is probably due to a genetic mutation or rare antibody response following multiple pregnancies or repeated transfusions. Genetically, this would require the union of a male hemophiliac with a carrier female and such a combination could be expected to be lethal to the ovum.

Common clinical findings include hemarthrosis and deformities caused by subcutaneous and intramuscular hematomas and bleeding into joints following slight trauma. Hematomas in the extremities may lead to secondary gangrene. Also with minor lacerations, there may be severe oozing of blood,

SECTION IV - ANNEXES

ANNEX C

BASIC PROCEDURES IN IMMUNOHEMATOLOGY AND BLOOD BANKING

OBJECTIVE: Given prepared specimens, consistently perform each of the fundamental blood banking procedures, to include ABO groups, Rh types, antiglobulin tests, and compatibility testing with an acceptable degree of accuracy when compared to the established factors in each specimen.

Subject and LP No		Lesson Objective	References
ABO Grouping and Confirmation Tests 08-280-100		Define the terms "antigen" and "antibody." List the antigens and antibodies of the four ABO blood groups. Explain the difference between cell grouping and confirmation testing (serum grouping). After practice, determine the ABO group of blood specimens with acceptable accuracy.	TM 8-227-3, pp 4-1 thru 4-20.
Rh Typing and Du Determination 08-280-102	I	Using the Wiener and/or Fisher-Race systems of nomenclature, list the five basic Rh antigens. Distinguish by definition between "phenotype" and "genotype." Explain the clinical importance of Rh sensitization. Discuss the difference between Rh negative and Du positive individuals. List and explain the steps in the performance of Rh_o determinations.	TM 8-227-3, pp 5-1 thru 5-20.
Antihuman Globulin Tests 08-280-104	I	Discuss the principles on which the direct and indirect antihuman globulin tests are based. Explain the difference between the direct and indirect applications of the antihuman globulin test and list situations where each may be used. Discuss the significance of both positive and negative reactions in the direct and indirect antihuman globulin tests.	TM 8-227-3, pp 7-1 thru 7-6.

Subject and LP No	Lesson Objective	References
Compatibility Testing 08-280-106	Discuss and explain the various phases of compatibility testing, with emphasis on interpreting results. List the various reactions encountered during and after the transfusion of whole blood. Discuss and explain emergency crossmatch procedures and urgent provision of donor blood.	TM 8-227-3, pp 11-1 thru 11-30.
Blood Donor Processing 08-280-108	Discuss the medical and physical criteria used in the selection of blood donors. Outline and explain the steps used in the procurement of blood from the donor. List and explain the various techniques and safety devices used in the storage, issue, and transportation of blood. Outline and explain the procedure used to detect the presence of atypical antibodies in donor blood. Explain the necessary steps in the processing of whole blood prior to use.	TM 8-227-3, pp 10-1 thru 10-32; VT 12.
Immunohematological Techniques 08-280-101	Using proper technique, perform tests of an immunohematological nature (ABO grouping, Rh_o (D) determination, direct anti-human globulin testing, indirect anti-human globulin testing, incomplete antibody detection, and compatibility test procedures) under laboratory conditions. Interpret results and report the various procedures in correct format during a proficiency survey.	All previous references.

Subject and LP No	Lesson Objective	References
Written Examination and Critique II 08-280-120	**Answer questions on theories, principles, and procedures presented in lecture. Demonstrate an ability to interpret the results of immunohematological test procedures given specific and example test data.**	All previous references.
Practical Examination and Critique II 08-280-119	Perform procedures practiced during the course **with attainment of an equivalent grade of at least 70 percent.**	All previous references.

SUPPLEMENTARY BIBLIOGRAPHY

American Association of Blood Banks. Technical Methods and Procedures of the American Association of Blood Banks. 5th ed. Chicago: American Association of Blood Banks, 1970.

Mollison, P. L. Blood Transfusion in Clinical Medicine. Oxford, England: Blackwell Scientific Publications, 1963.

Ortho Pharmaceutical Corporation. Blood Group Antigen and Antibodies. Raritan, New Jersey: Ortho Pharmaceutical Corporation, 1960.

Race, R. R., and Sanger, R. Blood Groups in Man. 5th ed. Philadelphia: F. A. Davis Company, 1968.

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M 08-280-111
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Immunohematology Lecture Objectives

The following is a list of immunohematology objectives, outlined per lecture as indicated, for the 311-92B10, Medical Laboratory Procedures (Basic) Course.

A. LP 08-280-100, ABO Grouping and Confirmation Testing

1. Define the terms "antigen" and "antibody."

2. Identify the antigens and antibodies of the four ABO blood groups.

3. Identify the difference between cell grouping and confirmation testing (serum grouping).

B. LP 08-280-102, Rh Typing and Du Determination

1. Identify the Rh antigen designations of Fisher-Race that correspond to those of Weiner.

2. Identify characteristics of the Rh antigens and antibodies to include those of the Du antigen.

3. Identify situations that can lead to Rh sensitization and the clinical importance of such sensitization.

4. Identify proper steps and possible sources of error in testing for the Rh antigens.

C. LP 08-280-104, Antihuman Globulin Tests

1. Identify the principles on which the direct and indirect antihuman globulin tests are based.

2. Identify applications of the direct and indirect antihuman globulin tests.

D. LP 08-280-108, Blood Donor Processing

1. Identify the medical and physical criteria used in the selection of blood donors.

2. Identify the proper steps used in the procurement of blood from the donor.

3. Identify the various techniques and safety devices used in the storage, issue and transportation of blood.

4. Identify the necessary steps in the processing of whole blood prior to use.

E. LP 08-280-106, Compatibility Testing

1. Identify the components of the major and minor sides of a crossmatch.

2. Identify the phases of compatibility testing, to include necessary reagents for each phase.

3. Identify the types of transfusion reactions.

4. Given the blood group and Rho type of both donor and recipient, predict possible crossmatch incompatibilities.

5. Identify the order of preference for selection of blood used for other than group specific transfusion.

F. LP 08-280-101, Immunohematological Techniques

Properly perform tests and procedures of an immunohematological nature, interpret results, record and classify results accurately and in correct format.

G. LP 08-280-119, Practical Examination and Critique II

1. Given prepared specimens, consistently perform ABO grouping, confirmation testing (serum grouping), Rho determinations and compatibility test procedures with a degree of accuracy equivalent to established guidelines.

2. Given prepared specimens, consistently perform direct and indirect antihuman globulin test procedures with a degree of accuracy equivalent to established guidelines.

H. LP 08-280-120, Written Examination and Critique II

1. Answer questions on theories, principles, and procedures presented in lecture, video TV tapes and reading assignments.

2. Interpret the results of immunohematological test procedures given specific and example test data.

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LABORATORY SCIENCES DIVISION 096
CLINICAL MICROSCOPY BRANCH

ABO Grouping and Confirmation Tests (2 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist Course

PLACE: Classroom, Bldg 2841

REFERENCES:

TM 8-227-3

STUDY ASSIGNMENT: Complete and return to class M 08-280-100-1. 014

STUDENT UNIFORM AND EQUIPMENT: N/A

TOOLS, EQUIPMENT, AND MATERIALS: None

PERSONNEL: One primary instructor (MOS 92B2H or 92B4H)

INSTRUCTIONAL AIDS: PA system with lapel microphone, chalk, chalkboard, and eraser

TROOP REQUIREMENTS: N/A

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Lecture

FIRST HOUR

I. INTRODUCTION (5 min)

A. Opening Statement: There are few procedures in the laboratory which deal as closely with the life and death of the patient as do the tests performed in the blood bank. A thorough understanding of the ABO blood group system is essential to the performance of compatibility tests. The work done in immunohematology must be mastered and must be without error. The life of the patient may literally be in your hands!

B. Objectives

1. Define the terms antigen and antibody.

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*This lesson plan supersedes LP 08-280-100, 014.

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2. Identify the antigens and antibodies of the four ABO blood groups.

3. Identify the difference between cell grouping and confirmation testing (serum grouping).

C. Class Procedure and Lesson Tie-in

1. First two hours of a 64 hour block of instruction.

2. Practical exercises to follow each lecture.

3. Conference when necessary to solve individual problems.

II. EXPLANATION (70 min)

A. Principles of Immunohematology

1. Study of antigen-antibody reactions in blood

2. Definitions

a. antigen (agglutinogen)

b. antibody (agglutinin)

c. specificity

d. avidity

e. titer

B. ABO Blood Group System

1. ABO antigen characteristics

a. Antigen on cell membrane

b. 2 antigens in ABO system: A and B

c. ABO blood group determined by presence or absence of antigen

1. A

2. B

3. AB

4. O

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2. ABO antibody characteristics
    a. Antibody present in serum or plasma
    b. Specific for antigen
        1. Anti-A -- A antigen
        2. Anti-B -- B antigen
    c. "Naturally" occurring 3-6 months after birth
    d. Reactions with antigen
        1. Type - agglutination
        2. Temperature
            a. Optimum - 4-6°C
            b. Performed at room temperature (20-25° C)
        3. Saline reactive - "complete" antibody

3. "Lansteiner's Rule" - if an ABO antigen is present on the cell, the corresponding antibody is absent from the serum.

NOTE: Put the following chart on chalkboard:

	Antigen A	Antigen B	Antibody A	Antibody B	ABO Group	% Occ
ABO Blood Group *in vivo*	Present	Absent	Absent	Present	A	40%
	Absent	Present	Present	Absent	B	10%
	Present	Present	Absent	Absent	AB	5%
	Absent	Absent	Present	Present	O	45%

NOTE: in explaining chart, re-emphasize "Landsteiner's Rule."

4. Genetics of the ABO system
    a. ABO genes
        1. A and B co-dominant

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2. O is amorphic

b. Genotype

   1. Homozygous

   2. Heterozygous

c. Phenotype

SECOND HOUR

C. Procedures

1. Cell grouping

   a. Principle - in vitro reaction between unknown antigen and known specific antibody

   b. Reagents

      1. Source - humans

         a. Anti-A - from group B persons

         b. Anti-B - from group A persons

         c. Anti-A,B - from group O persons

      2. Color coded

         a. Anti-A - blue

         b. Anti-B - yellow

         c. Anti-A,B - normal clear serum

   c. Methods

      1. Slide method

         a. Use one clean microscope slide

         b. Label one side A, the other B

         c. Place specimen number or patient's name on top

         d. Place 2 drops anti-A on A side

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e. Place 2 drops anti-B on B side

f. Using two applicator sticks, put one drop blood on each side and mix with antisera

g. Read for agglutination (negative only after 2 min)

2. Tube method

a. Prepare a 2-5% cell suspension

  1. Fill a tube 3/4 full with saline

  2. Dip two applicator sticks into blood

  3. Transfer to saline tube and mix

  4. Suspension should be cherry-red

b. Suspension should be washed 4-6 times prior to use.

c. Label two 10x75 mm test tubes A & B respectively

d. Place specimen number or patient's name on each tube

e. Put 2 drops anti-A into A tube

f. Put 2 drops anti-B into B tube

g. Put 2 drops of cell suspension into each tube

h. Mix blood and antisera

i. Spin in Serofuge for 15 seconds

j. Read for agglutination

k. If both tubes are negative, test the cells with anti-A,B

  1. Anti-A,B used to confirm persons of group O

  2. Anti-A,B will agglutinate cells of groups A, B, AB, and subgroups

  3. To run test, repeat steps "a" thru "j", using one tube with 2 drops of anti-A,B

d. Interpretation

1. Agglutination - antigen present on cells (+)

2. No agglutination - antigen absent from cells (-)

NOTE: Place following chart on chalkboard:

Unknown Cells in vitro reaction with:	Anti-A	Anti-B	Antigen Present	ABO Group
	+	-	A	A
	-	+	B	B
	+	+	A and B	AB
	-	-	None	O

NOTE: In explaining chart, re-emphasize that the ABO group is determined by the presence or absence of A and B antigen on the cells.

2. Confirmation tests (serum grouping)

a. Principle - in vitro reaction between known antigens and unknown antibodies

b. Reagents

1. 2-5% saline suspension of known group A cells

2. 2-5% saline suspension of known group B cells

c. Method

1. Label two 10x75 mm test tubes A & B respectively

2. Place specimen number or patient's name on tubes

3. Place 2 drops of unknown serum in each tube

4. Place 1 drop saline suspension of A cells in A tube

5. Place 1 drop saline suspension of B cells in B tube

6. Mix serum and cells

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7. Spin in Serofuge 15 seconds

8. Read for agglutination

9. If serum group does not confirm cell group, retest, then refrigerate only negative tests

d. Interpretation

1. Agglutination - antibody present in serum (+)

2. No agglutination - antibody absent from serum (-)

NOTE: Place following chart on chalkboard:

	A Cells	B Cells	Antibody Present	Antigen on Cells from specimen	ABO Group
Unknown Serum under study	-	+	anti-B	A	A
	+	-	anti-A	B	B
	-	-	None	A & B	AB
	+	+	Both	None	O

QUESTION: Why is the serum grouping called a confirmation test?

ANSWER: By cell grouping with known antiserum, we have defined the ABO group of a specimen, since this tells us which of the antigens are present or absent. We know that if a particular antigen is present, the corresponding antibody should not be found in the serum, so we test the serum with known antigens. If our testing of the serum shows that the corresponding antibody is absent, we have confirmed the results of the cell grouping test.

D. Sources of Error

1. Failure to follow manufacturer's instructions

2. Improper proportions of cells and serum

3. Improper centrifugation

4. Contaminated reagents

5. Improper interpretation or reading of results

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6. Dirty glassware

E. NIH Standards for Reagents

1. Avidity

a. Anti-A - agglutination of group A cells must begin in no more than 15 seconds

b. Anti-B - agglutination of group B cells must begin in no more than 15 seconds

2. Titer - concentration of antibodies

a. Expressed as reciprocal of dilution

b. 1:256 for broth anti-A and anti-B

c. No minimum requirement for anti-A,B

3. Specificity

a. anti-A only reacts with A antigen

b. anti-B only reacts with B antigen

F. Questions from Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Principles of Immunohematology

2. ABO Blood Group System

3. Procedures

B. Closing Statement: The accuracy with which the procedures are done in immunohematology cannot be over-stressed. As a technician working in the blood bank section, the well-being of a patient depends upon the quality of your work. There is no room for error!

NOTE: Hand out H 08-280-100-1; 014, one per student.

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*IM 08-280-100
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ABO Grouping and Confirmation Tests

There are few procedures in the laboratory which deal as closely with the life and death of the patient as do the tests performed in the blood bank. A thorough understanding of the ABO blood group system is essential to the performance of compatibility tests. The working done in immunohematology must be mastered and must be without error. The life of the patient may literally be in your hands!

Before attempting the procedures performed in immunohematology, you should know some of the principles involved. Stated simply, immunohematology is the study of antigen-antibody reactions in blood. These two basic entities must now be defined. An antigen (agglutinogen) is a substance, usually protein in nature, which, when introduced into the body of an animal lacking that substance, stimulates the production of antibodies specific for that substance. Conversely, an antibody (agglutinin) is a substance globulin in nature, produced as a result of stimulus by an antigen. The antibody reacts only with the antigen which stimulated the body to produce it. This antigen-antibody reaction is called specificity. The speed of such antigen-antibody reactions is called avidity. One more term you should be familiar with is "titer." Titer is the concentration of antibodies expressed as the reciprocal of the dilution. The higher the titer, the higher the concentration of antibodies.

In order for you to understand the ABO blood group system, we must first discuss the antigens and antibodies which comprise the system. First, we will discuss the ABO antigens. These antigens are found on the erythrocyte cell membrane as early as two months after conception. The ABO system is comprised basically of two antigens: "A" and "B." The ABO group of an individual is determined by the presence or absence of these antigens. A person who is blood group "A" would possess the antigen "A." Group "B" individuals have the "B" antigen. Group "AB" individuals have both "A" and "B" antigens. Group "O" individuals lack both antigens.

The ABO antibodies are present in serum or plasma. They are specific for their corresponding antigen. Anti-A is specific for the "A" antigen and anti-B is specific for the "B" antigen. ABO antibodies are "naturally" occurring and appear 3-6 months after birth. By "naturally" occurring, we mean that they are formed as a response to antigens found in or on plants, animals, and micro-organisms which are similar to the "A" and "B" antigens. "Immune" antibodies on the other hand, are usually produced by an individual as a result of some known antigenic stimulus, such as an incompatible transfusion, pregnancy, or by injection of blood group substances or antigenic material.

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*This instructor's manuscript supersedes IM 08-280-100, 014.

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The typical reaction of the ABO antigens and antibodies is agglutination, or the clumping together of cells. The optimum temperature range for this reaction is 4-6° C. However, the antibodies will also react well at room temperature (20-25° C). This is advantageous to the technician in that no refrigeration is necessary to routinely perform these tests. The antibodies react well in a saline medium, since they are relatively long and can bridge the gap between cells. They are termed "complete" antibodies.

Dr. Karl Landsteiner is credited with the discovery of the four blood groups in man. He discovered that the cells of certain persons would agglutinate in the serum of others. On the basis of his observations, he divided these individuals into three blood groups according to the antigens theorized to be present on the cells. He assigned the letters "A", "B", and "O" to these individuals. Two of his students, DeCastello and Sturli, discovered the fourth blood group, "AB". On the basis of these findings he formulated the axiom which is today known as "Landsteiner's Rule" -- if an ABO antigen is present on the cell, the corresponding antibody is absent from the serum. This concept must be understood in order to study immunohematology successfully.

A brief discussion of the genetics of the ABO blood group system is in order. ABO groups are determined by the genes "A", "B", and "O". The "A" and "B" genes are co-dominantly expressed on the chromosome loci. This means that if an "A" gene is on one chromosome locus and the "B" gene on the other chromosome locus, both will show up in the person's physical characteristics. The "A" gene will produce "A" antigen, while the "B" gene will produce "B" antigen. The "O" gene is an amorphic allele. It causes the production of no antigen. In other words, it is the lack of the "A" and "B" antigen.

Since chromosomes are found in pairs in somatic (body) cells, there are two gene sites on which either the "A," "B," or "O" gene may be found. These genetic pairs are called the person's genotype. Genotypes are written as AO, AB, AA, BO, BB, OO, etc. A person who inherits like genes is said to be homozygous for the trait while if a person inherits unlike genes he is said to be heterozygous for the trait.

In determining an ABO blood group one does not see what is on the genes, but what is physically expressed in the body. This physical expression or characteristic is called phenotype. The four phenotypes found in the ABO blood group system are "A," "B," "AB," and "O".

Now that the basic theories behind the ABO blood group system have been discussed, we will talk about the procedures involved. The first is the cell grouping. The principle behind cell grouping is an "in vitro" reaction between unknown antigens and known specific antibodies. To perform this procedure, several reagents will be required. These reagents are obtained from human donors and sold by commercial companies. Anti-A is obtained from group "B" persons. Anti-B is obtained from group "A" persons. Anti-A,B is obtained from group "O" persons. These reagents are color coded as prescribed by the NIH (National Institute of Health). Anti-A is blue, anti-B is yellow, and anti-A,B has the appearance of normal, clear serum.

There are two methods of performing the ABO cell grouping -- the slide and test tube methods. Of these, the test tube method is the most commonly employed. First, we will discuss the slide method.

To perform the slide method we must have one clean microscope slide and an indelible marker. First, label one side of the slide "A", and the other side "B". Place the specimen or patient's name on top. Place 2 drops of anti-A on the "A" side, approximately in the middle of the slide. Next, place 2 drops of anti-B on the "B" side. Using two applicator sticks, put one drop of blood on each side. Use a different applicator stick for each side of the slide. Mix the blood and antiserum until the mixture is about the size of a nickle. Tilt the slide back and fourth while observing for agglutination. Agglutination will appear as a clumping of cells. No agglutination will appear as a smooth, homogenous mixture of cells and antiserum. The tilting should be done for a minimum of 2 minutes.

The test tube method of cell grouping is performed more routinely. To perform this procedure properly, a 2-5% cell suspension is required. To prepare the cell suspension, first fill a test tube 3/4 full with saline. Dip two applicator sticks into the blood sample and transfer to the saline filled tube. Mix by swirling the applicator sticks. If the suspension is made properly it will be cherry-red in color. The cell suspension should be washed 4-6 times prior to use to insure removal of hemolyzed cells and excess protein that may interfere with the test.

Once the cell suspension is made, label two 10x75 mm test tubes "A" and "B" respectively. Place the specimen number or patient's name on each tube. Put two drops of anti-A into the tube labeled "A," and two drops of anti-B into the tube labeled "B". Next, add two drops of the 2-5% cell suspension to each of the tubes. Adequately mix the blood and antisera and then spin in the Serofuge for 15 seconds. Once the specimens have been spun, read them for macroscopic agglutination. If both of the tubes are negative for agglutination, the cells should then be tested with anti-A,B grouping serum.

Anti-A,B is used in confirmation testing of all individuals of blood group "O" because it will agglutinate all red cells of blood groups "A", "B," "AB," and subgroups. The true nature of anti-A,B is open to discussion, although it is thought to be a true combination antibody that can detect subgroups. To perform the anti-A,B test, the test tube method is used. To a test tube containing two drops of the cell suspension, two drops of anti-A,B are added. From this point on, this procedure is the same as that used in the test tube ABO cell grouping technique.

Now that the unknown cells have been tested with known antibodies, an interpretation of the results is necessary. If agglutination is seen, the antigen for that specific antibody is present on the cells, and is reported as positive (+). If no agglutination is seen, the antigen is absent from cells and is reported as negative (-). We must keep in mind that the ABO group of a blood specimen is defined by the presence of "A" and "B" antigens on the cells, as determined by positive or negative reactions with known antibodies. The cells giving agglutination with anti-A, but no reaction with anti-B, must contain the antigen "A". Its cell group must be "A". Cells which are negative with anti-A but are agglutinated by anti-B contain the "B;" therefore, the blood group must be "B". If agglutination has occurred with both anti-A and anti-B, both antigens must be present, and the blood group is "AB". So far, we have considered procedures where reactions have taken place with either or both antisera. Now, let us take the last case where the cells remain smoothly suspended with no agglutination present. Here we find neither "A" nor "B" antigen on the cells. This being true, we then know that the cells are of blood group "O". As stated earlier, to confirm this one must also test these cells with anti-A,B antisera. If this test is also negative, then the person is truly a group "O" individual.

The application of "Landsteiner's Rule" gives us a procedure known as confirmation testing, more commonly called "serum grouping". The principle of confirmation testing is quite simple. It consists of an "in vitro" reaction between known antigens and unknown antibodies.

The reagents used in confirmation testing are a 2-5% saline suspension of known group "A" cells, and a 2-5% saline suspension of known group "B" cells. Although the slide method may be used in this procedure, the test tube method is preferred. First, label two 10x75 mm test tubes "A" and "B" respectively. Place the specimen number or patient's name on the tubes. Then put two drops of that unknown serum into each tube. Place one drop of the saline suspension of "A" cells into the tube labeled "A", and one drop of the saline suspension of "B" cells into the tube labeled "B". Mix the serum and cells thoroughly. Place both tubes in the Serofuge and spin for 15 seconds. Upon completion of the spinning phase, remove the tubes from the Serofuge and read them for macroscopic agglutination.

If the results of the confirmation test do not confirm those results previously obtained in the cell grouping technique, it is necessary to rerun both procedures. If they still do not confirm, place only the negative tests in the refrigerator for approximately 5-10 minutes. Remember that the optimum temperature for the ABO antigens and antibodies to react is 4-6° C. At the end of the refrigeration, spin the tubes in the Serofuge for 15 seconds and read them for macroscopic agglutination.

The results obtained should now be interpreted interms of ABO groups. If agglutination occurs, this means that the antibody is present in the serum, whereas if no agglutination occurs, the antibody is absent from the serum. If the unknown serum agglutinates the group "B" cells, but not the group "A" cells, anti-B is present in the serum. According to "Landsteiner's Rule", this blood specimen must be group "A". If group "A" cells are agglutinated, but the "B" cells are not, the serum contains anti-A and the specimen is group "B". When neither of the known cells agglutinate, then neither antibody is present. In this case, the blood group is "AB". If both the known test cells are clumped, both antibodies are present and the specimen is group "O". By placing side-by-side properly labeled reaction charts of the cell grouping and serum grouping procedures, you can see that each reaction is the reverse of the other for each blood group. This easily understandable if you keep in mind that the antigen on the cell cannot have the corresponding antibody present in the serum of the same blood specimen.

As with any procedure in the laboratory, there are sources of error which must be avoided. The first of these is the technician's failure to follow the manufacturer's instructions for respective determinations which are to be made. These procedures have been tested extensively, and have proven to work. Any deviation could result in erroneous test results. Improper proportions of cells and serum should also be avoided. Too many cells could result in agglutination that is not visible macroscopically, and as a result, cause the test to be reported as negative. Centrifugation times should also be strictly adhered to. Excessive centrifugation can mechanically clump the cells together, giving the appearance of a positive. Contaminated reagents, either by the mixing of different antisera, or from bacterial growth, should be discarded. Reading of tests is to be mastered before reporting results. Glassware should be scrupulously clean, as residual serum from previous tests and certain chemicals could interfere with reactions.

There are other NIH standards for reagents which should be known concerning antisera. The avidity of both anti-A and anti-B must be such that agglutination of their specific cells begins in no more than 15 seconds. The titer or concentration of antibodies in the antiserum for both anti-A and anti-B is at least 1:256. There is no minimum titer for anti-A,B. The specificity is such that anti-A reacts only with the "A" antigen and anti-B reacts only with the "B" antigen.

The principles of immunohematology have been discussed. The ABO blood group system has been discussed in detail, to include the ABO antigen and antibody characteristics; "Landsteiner's Rule", and the associated genetics which are involved. The procedures for performing both cell and serum grouping have been explained.

The accuracy with which the procedures are done in immunohematology cannot be over-stressed. As a technician working in the blood bank section, the well-being of a patient depends upon the quality of your work. There is no room for error!

PFC Judity Atchison

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
PATHOLOGY AND LABORATORY SCIENCES DIVISION
HEMATOLOGY BRANCH

*M 08-280-100-1
014

Study Assignment

Cell and Serum Grouping

Following questions are to be answered after class and returned to your instructor at the time specified. You may use your notes and available reference material.

1. On four blood specimens for ABO grouping you have obtained the results shown below. Place the blood group of each specimen in the blank.

	Cell Grouping		Serum Grouping		
Specimen	Anti-Serum		Known Cells		Blood Group
	Anti-A	Anti-B	A Cells	B Cells	
1	0	0	+	+	______
2	+	0	0	+	______
3	+	+	0	0	______
4	0	+	+	0	______

2. Commercially prepared anti-A is obtained from Group _____ persons; anti-B is obtained from Group _____ persons. Commercial anti-A is _____ in color, while anti-B is colored _______. To be sold commercially antisera must meet or exceed the National Institute of Health (NIH) requirements on _______, _______, and _______.

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*This mimeo supersedes M 08-280-100-1, 061.

3. In immunohematology an antigen may be called a/an ____________, while an antibody may be known as a/an ____________.

4. Landsteiner found that when an antigen is present in the blood the corresponding antibody is absent, while the opposite antibody is usually present. Assuming this to be correct, if you have demonstrated the presence of A antigen you would expect to find __________ antibody.

5. Generally speaking the antigens and antibodies of the ABO blood group system are present in the blood. Specifically, the antigens are found in or on the _________, while antibodies are found in the ________ or ________.

6. Confirmation testing (also called "serum-grouping") utilizes the patient's __________, and known __________, so that we are checking the ____________ content of the patient's blood.

7. In performing the cell grouping we use the patient's ____________ combined with known ____________, so that we are testing for the _________ content of the patient's blood.

8. In order to meet NIH requirements ABO antisera must have a titer of at least 1:__________, and avidity must be such that reaction begins no more than ________ seconds after cells and antiserum are brought together.

9. ABO antibodies react well in saline, and cells are usually tested after suspending them in isotonic saline to a concentration of ________ to ________ percent.

10. In ABO grouping, you may occasionally see false agglutination, in which the cells under the microscope take on the appearance of stacks of coins. This phenomenon, known as ___________ ___________ can usually be prevented by washing the cells in __________ prior to testing.

11. The naturally occurring antibodies of the ABO system are not usually demonstrable until the patient is _________ to __________ of age.

12. Since ABO antibodies are "cold reacting," heating the antigen-antibody test mixture is a major source of error in ABO grouping. For this reason the test is usually performed in a temperature range of _________° C. to ____________° C.

13. Define "antigen."

14. Define "antibody."

15. __________ is a visible reaction which takes place when, under the proper conditions, cells containing specific __________ are mixed with the corresponding ________________.

16. Tests on the cells of a blood specimen show that specimen to be Group A; however, serum grouping indicate the specimen belongs to Group AB, since no antibodies can be detected. What would be your first step in resolving this discrepancy?

17. You have received a blood specimen for ABO grouping and are told that the patient is a newborn infant. Explain why only cell grouping need be performed.

18. When performing ABO cell grouping tests on a slide, the test may not be considered negative unless no agglutination has appeared in ______ minutes.

19. A person inherits two genes for each physical characteristic, one from each parent. If these genes are alike we say that the person is ________ for that trait; if the genes are different, the person is said to be ____________.

20. A person's blood group is defined by the presence or absence of the ABO __________ in/on his ____________.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-102
LABORATORY SCIENCES DIVISION 024
HEMATOLOGY BRANCH

Rh Typing and D^u Determination (2 hours)

COURSES PRESENTED TO: 311-92B20 Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES:

TM 8-227-3
American Association of Blood Banks, Technical Methods and Procedures of the American Association of Blood Banks, 5th ed. Chicago: American Association of Blood Banks, 1970.

STUDY ASSIGNMENT: After class, study notes taken during lecture.

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT AND MATERIALS: None

PERSONNEL: One primary instructor (MOS 92B4H or 92B3H)

INSTRUCTIONAL AIDS: PA system with lapel microphone, chalk, chalkboard and eraser.

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

FIRST HOUR

I. INTRODUCTION (5 min)

A. Opening Statement: An article published in a scientific journal was given wide coverage by the press and popular magazines in 1940. It caused a state of near panic in a large segment of the population. This article, by Dr. A. S. Wiener, concerned the discovery of the Rh antigen. Distortions published in many magazines since the discovery of the Rh factor influenced practically every young woman of child-bearing age in thinking that any child she might have would be erythroblastic. In the population at large, this lack of knowledge has persisted to some extent to the present time. However, in the clinical laboratory, as in other fields of scientific endeavor, half-truths, distortions of fact, and superstitions cannot be tolerated. Fortunately, advances in knowledge of the Rh blood group system enable the blood

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*This lesson plan supersedes LP 08-280-102, 071.

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bank technician to base his work in this area on facts, rather than on superstition and ignorance.

B. Objectives

1. Recall and list (using the Wiener and/or Fisher-Race systems of nomenclature) the five basic Rh system antigens.

2. Distinguish, by definition, between "phenotype" and "genotype."

3. Explain the clinical importance of Rh sensitization.

4. Discuss the difference between Rh negative and D^u positive individuals.

5. List and explain the steps in the performance of Rh_o determinations.

C. Class Procedure and Lesson Tie-in

1. Two hours on Rh typing and D^u determinations. This lecture is the second block of a total of 64 hours of instruction, practice, and examinations on theories, principles, and performance of routine pretransfusion tests and the standard 3-phase compatibility test.

2. One hour demonstration and practical exercise in accordance with individual needs to follow lecture, with minimum practice of 3 hours.

3. Conference when necessary to solve individual problems.

II. EXPLANATION (80 min)

A. Rh_o System Characteristics

1. Antigen (specific for its antibody) on erythrocyte

2. Reacts best at body temperature (37° C)

3. Reacts best in high protein medium

4. Separate from ABO groups

5. Normally no natural antibody

B. Rh Blood Group Nomenclature

1. Two systems

   a. Wiener

   b. Fisher-Race

2. Comparison and equivalents

   a.

Wiener	Fisher-Race
Rh_o	D
rh'	C
rh''	E
hr'	c
hr''	e
-	d (?)

   b. d antigen

      (1) Existence theoretical

      (2) Reciprocal relationship to Rh_o (D) postulated

QUESTION: Why is it necessary for a worker in the laboratory to learn both systems of nomenclature?

ANSWER: Although the Fisher-Race system is generally used in the laboratory, antisera will be labeled with both systems, while articles and reference books may use either or both systems.

C. Genetics

1. Basic elements

   a. Chromosomes

   b. Genes

2. Mechanism

   a. Chromosomes (human)

(1) Somatic cells (46)

(2) Germ cells (23)

b. Gene for each characteristic

(1) One from each parent

(2) Homozygous (like genes)

(3) Heterozygous (unlike genes)

(a) Allele

(b) Locus

c. Relationship of D/d antigen

(1) Genes for blood groups co-dominant

(2) "d̲" an amorphic gene and/or non-existent

(3) Phenotype

(a) Definition

(b) Application to Rh system

(4) Genotype

(a) Definition

(b) Application to Rh system

NOTE: Use chalkboard to demonstrate phenotypes and genotypes (see Annex A).

QUESTION: What will be the phenotype of children born to a homozygous Rh_o (D) father and a homozygous d̲ mother? What will be the genotype of these children?

ANSWER: These children will all be Rh positive (phenotype), since only Rh_o antigen can be recognized. The children will all be heterozygous because each has two different genes for the Rh factor, one from each parent.

QUESTION: Give the phenotypes and genotypes of children born to a heterozygous Rh positive father and a homozygous Rh negative mother.

ANSWER: Fifty percent of the children will be Rh positive and fifty percent Rh negative (phenotype). The Rh positive children will be heterozygous, having both genes; the Rh negative children will be homozygous since they inherit only d genes (genotype).

QUESTION: Is it possible for two Rh positive parents to have Rh negative children?

ANSWER: Yes. If both parents are heterozygous, one-fourth of the children may be Rh negative.

D. Sensitization to Rh_o (D) Antigen

NOTE: We have discussed only the Rh_o (D) antigen. Antibodies specific to this antigen are not naturally occurring (excepting rare cases).

1. Causes

a. Blood transfusions

QUESTION: May we give Rh positive blood to an Rh negative patient?

ANSWER: This is definitely not common practice and should be done only in extreme emergencies, since we will probably sensitize the patient, with resultant production of antibodies.

b. Pregnancy

QUESTION: Can a person be sensitized and produce antibodies against Rh antigen by means other than transfusion of blood?

ANSWER: Yes. Pregnancy can result in sensitization. If an Rh negative woman carries an Rh positive fetus, cells from the fetus cross the placental barrier into the mother's circulation, causing her to produce antibodies against the Rh_o (D) antigen.

QUESTION: Since the woman lacks the antigen, no reaction takes place; why is the circulating antibody important?

ANSWER: Because this antibody from the mother's serum recrosses the placental barrier into the circulation of the fetus where it reacts with the Rh positive cells, hemolyzing large numbers and causing a condition known as hemolytic disease of the newborn (HDN).

c. Deliberate

2. Antibodies produced

a. "Complete" (saline-active)

b. "Incomplete"

(1) Albumin

(2) Antihuman globulin (AHG)

E. Rh_o (D^u) Variant

1. Nature of antigen

a. Doubtful or negative with saline-active anti-Rh_o (D)

b. Positive by albumin or AHG techniques

2. Importance: causes sensitization

SECOND HOUR

F. Rh_o (D) Determination

1. Principle: to detect Rh_o (D) antigen

2. Reagent: anti-Rh_o (D)

a. Source: sensitized human donors

b. Titer: 1:32

c. Specificity: specific for Rh_o (D)

3. Methods

a. Slide method

(1) Equipment

(a) Glass slides

(b) View box (45° - 50° C)

- (c) Indelible marker
- (d) Applicator sticks

(2) Procedure

- (a) Label slide with patient's identification
- (b) Place 2 drops anti-D on slide
- (c) With 2 applicator sticks, place 1 drop patient's blood next to drops of anti-D
- (d) With applicator stick, mix anti-D and blood until most of slide is covered
- (e) Place on view box and tilt 2 minutes
- (f) Read for agglutination at 2 minutes

(3) Interpretation

- (a) Agglutination
  - 1 Positive test result
  - 2 Rh positive
- (b) No agglutination
  - 1 Negative test result
  - 2 Further testing required
    - a Test tube method
    - b D^u (if test tube method is negative)

b. Test tube method

(1) Equipment

- (a) Test tubes (2)
- (b) Serofuge
- (c) Indelible marker

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(d) Applicator sticks

(e) Bovine albumin (control)

(2) Procedure

(a) Label 2 tubes with patient's identification, one tube "D" and one "Con"

(b) To "Con" add 2 drops bovine albumin

(c) To "D" add 2 drops anti-D

(d) Using one applicator stick, transfer patient's cells to control ("Con") tube

(e) Using one applicator stick, transfer patient's cells to "D" tube

(f) Serofuge 15 seconds

(g) Read for agglutination

(3) Interpretation

(a) Agglutination

1 Positive test result

2 Rh positive

(b) No agglutination

1 Negative test result

2 Proceed to perform D^u determination on both tubes

c. D^u method

(1) Reagent: antihuman globulin

(2) Procedure (follow steps a-g of Rh_o determination)

(a) Resuspend cell button

(b) Incubate both tubes 15 minutes at 37° C.

(c) Wash 4 times with saline

(d) Decant supernatant

(e) Resuspend cell button

(f) Add 2 drops antihuman globulin

(g) Serofuge 15 seconds

(h) Read for agglutination

(3) Interpretation

(a) Agglutination

1 Positive test result

2 D^u (Rh_o) positive

(b) No agglutination

1 Negative test result

2 Rh negative

G. Sources of Error

1. Rouleaux formation
2. Drying of cell-serum mixture (slide)
3. Contamination of antiserum
4. Improperly cleaned glassware
5. Excess anticoagulant used
6. Improper cell-antibody ratio
7. Failure to follow manufacturer's instructions

H. Questions From Students

III. SUMMARY (5 min)

A. Review of Main Points

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1. Rh_o system characteristics
2. Rh blood group nomenclature
3. Genetics
4. Sensitization to Rh_o (D) antigen
5. Rh_o (D^u) variant
6. Rh_o (D) determination
7. Sources of error

B. Closing Statement: In your future assignments as laboratory technicians, you will be performing countless Rh and D^u determinations. Never let this task become routine! In this, as in any other laboratory procedure, your understanding of the principles and meticulous attention to details will enable you to report accurate and timely results. These results—the end point of your work—will determine the success or failure of your mission.

ANNEX A
INSTRUCTIONAL AIDS

	D	D
d	Dd	Dd
d	Dd	Dd

Father: homozygous Rh_o (D)
Mother: homozygous Hr_o (d)
Children: phenotype Rh positive
genotype Dd

	D	d
d	Dd	dd
d	Dd	dd

Father: heterozygous Rh_o (D)
Mother: homozygous Hr_o (d)
Children: 50 percent Rh positive (phenotype), with genotype Dd; 50 percent Rh negative (phenotype) with genotype dd

	D	d
D	DD	Dd
d	Dd	dd

Father: heterozygous Rh positive
Mother: heterozygous Rh positive
Children: one-fourth of children will inherit only Hr_o (d) genes and will be Rh negative

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HEMATOLOGY BRANCH

Rh Typing and D^u Determination (2 hours)

An article published in a scientific journal was given wide coverage by the press and popular magazines in 1940. It caused a state of near panic in a large segment of the population. This article, by Dr. A. S. Wiener, concerned the discovery of the Rh antigen. Distortions published in many magazines since the discovery of the Rh factor influenced practically every young woman of childbearing age in thinking that any child she might have would be erythroblastotic. In the population at large, this lack of knowledge has persisted to some extent even to the present time. However, in the clinical laboratory, as in other fields of scientific endeavor, half-truths, distortions of fact, and superstitions cannot be tolerated! Fortunately advances in knowledge of the Rh blood group system enable the blood bank technician to base his work in this area on facts, rather than on superstition and ignorance.

In 1939, Dr. Levine and Dr. Stetson found an immune serum in a woman who had just delivered a stillborn fetus. She had a severe transfusion reaction from receiving a unit of her husband's blood. It was found that her serum agglutinated her husband's cells, as well as those of some 85 percent of other ABO compatible donors. The two workers theorized that the fetus had inherited from its father a blood factor. This antigen-antibody combination, they decided, had been responsible for the child's death before birth. The later course of events proved them right in this theory.

In 1940, Dr. Karl Landsteiner (who had 40 years earlier discovered the ABO blood groups) and Dr. A. S. Wiener began the studies which led to the definition of the Rh blood group system. Feeling that antigens similar to as yet unknown human antigens might be present in more pure form in the blood of Rhesus monkeys, they injected rabbits with monkey cells. Using the immune serum from the rabbits, they found that 85 percent of Caucasian blood specimens tested were agglutinated, even after known antibodies were absorbed. The remaining antibody they named anti-Rh, after the Rhesus monkey whose cells had stimulated its production. The antigen recognized by the antibody was called Rh. That there was no relation to ABO or other known blood groups was shown by the fact that the test cells were agglutinated in the same 85 to 15 percent proportions irrespective of other antigens known to be present on the cells. Persons whose cells reacted were called Rh positive; those whose cells failed to react were presumed to lack the antigen and were called Rh negative. Later studies showed that the Rh antibody of Landsteiner and Wiener was similar in specificity to antibodies found in the serum of persons who had reactions after the transfusion of ABO compatible blood. It was also similar to the antibody in the serum of the woman originally studied by Levine and Stetson, and the relationship between hemolytic disease of the newborn (HDN) and Rh incompatibility was established.

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*This instructor's manuscript supersedes IM -08-280-102, 071.

Puzzling cases began to appear, cases in which anti-Rh could not be found in the serum of mothers of clinically diagnosed HDN babies. In the light of our present knowledge, this is understandable since the searches for the antibody were conducted by using only saline-suspended cells. It was about this time that the terms "complete" (describing antibodies reactive in saline) and "incomplete" (describing antibodies reactive in protein or antihuman globulin) came into being. Dr. Diamond and Dr. Abelson found that if the cells were suspended in a medium high in protein concentration (plasma, serum, or albumin) the "incomplete" antibodies, previously looked for in vain, would react (optimally at body temperature, 37° C).

During the next 2 or 3 years, other factors in the Rh system were discovered, including reciprocal or related factors in "Rh negative" individuals. The list of known factors has grown over the years until what was once considered a simple system of Rh positive and Rh negative has become one of the most complex of all the known blood group systems.

At the present time are two nomenclatures routinely used in naming the antigens contained in the Rh blood group system. In this country Dr. Wiener, who with Landsteiner named the system Rh, recommends the use of the symbols Rh$_o$, rh', rh'', hr', an hr''. In England, Sir Ronald Fisher and Dr. R. R. Race evolved what they considered to be a simple system to designate the major antigens. Their nomenclature uses only the letters C, D, E, c, d, and e for the antigens which make up the basic Rh blood group system. Dr. Wiener's system, with the Fisher-Race equivalents, are as follows:

Wiener	Fisher-Race
Rh$_o$	D
rh'	C
rh''	E
hr'	c
hr''	e
-	d

We have said that the existence of d is theoretical. This is because an antigen may be recognized and defined only by an observable reaction with a specific antibody. Each of the first five of the basic Rh antigens was so recognized, but no antibody has yet been found for the antigen d. With the other antigens a reciprocal relationship seems to exist, i.e., in the absence of rh' (C) the presence of hr' (c) can be shown, and so on. For this reason, in spite of the fact that no antibody is available to prove this theory, the presence of d in the absence of Rh$_o$ (D) is assumed by Fisher-Race. A worker in the laboratory field must have knowledge of both nomenclature systems because the antisera may be labeled with both systems, while scientific articles and reference books may use either or both systems. In this course we will concern ourselves only with the antigen Rh$_o$ (D) since it seems more highly antigenic, thus making it of more clinical significance.

The inheritance of Rh$_o$ (D) and other blood factors is governed by the basic genetic elements. Within the nuclei of living cells are dark-staining, rod-shaped bodies called chromosomes. The chromosomes are thought to be made up of minute units, the genes. A gene, the basic unit of inheritance, is an entity which causes one inheritable characteristic to be carried from parent to child. These ganes are arranged in linear fashion within the chromosome. EACH gene determining the inheritance of a PARTICULAR trait or characterisitc occupies a particular position (locus) on the chromosome.

Each species has a standard number of chromosomes. In Man, each of the somatic (body) cells has 46 chromosomes arranged in pairs. The mature germ cells, (sperm and ova) with which we are concerned, have 23 chromosomes each. It can be seen, then, that fertilization of the ovum (with 23 chromosomes) by the sperm (also with 23 chromosomes) gives us the species number of 46, or 23 pairs. An individual inherits one of each pair from his mother and the other from his father. He, in turn, passes on in a random manner one of each pair to his children.

Chromosomes of similar size and shape tend to align themselves together. A gene for a particular trait (i.e., hair or eye color) at a specific locus on a chromosome will therefore have an alternate gene (called "allele") present at the same locus on the partner chromosome. In general, it can be said that for each inheritable characterisitc, there are two allelic genes, one from each parent. When the genes are alike the condition is said to be HOMOZYGOUS for that characteristic; when the genes are unlike, the person is HETEROZYGOUS.

Characteristic of genes is their ability, or lack of it, to manifest themselves in the presence of their alleles. In the case of d and its postulated reciprocal relationship to Rh$_o$ (D), for instance, Hr$_o$ (d) does not express itself in the presence of Rh$_o$ (D). Because all blood group systems are said to be CO-DOMINANT, we term Hr$_o$ (d) AMORPHIC, in other words, since Rh$_o$ (D) and theorized d are CO-DOMINANT, d does not express itself in the presence of Rh$_o$ (D) simply because it (d) is amorphic. This leads us to the terms "phenotype" and "genotype." Phenotype refers to visible or demonstrable characteristics while genotype is the sum total of an individual's genes. In terms of the genes for the Rh factor, this distinction may be made clear with an example. A person whose cells are agglutinated in the presence of anti-Rh$_o$ (D) has the PHENOTYPE "Rh positive." Therefore we know he carries at least one gene for Rh$_o$. He may carry two genes for Rh$_o$ (d), or he may have the gene for its amorphic reciprocal d; that is, his GENOTYPE may be DD or Dd. In the absence of an antiserum for d (this is why its existence is only theorized), this person's genotype for Rh can be determined only by familial studies.

On the other hand, a person whose cells are NOT agglutinated by anti Rh_o (D) has the PHENOTYPE "Rh negative." In his case, we must assume a genotype of dd, i.e., he is homozygous for the gene d since the presence of Rh_o (D) would have been detected by the antiserum.

NOTE: Use chalkboard to demonstrate phenotypes and genotypes (see Annex A).

So far, we have limited our discussion to antigens of the Rh system; the presence of an anti-Rh in an individual is not a normal finding. Since Rh antibodies are not usually naturally occurring, it is an indication that the individual LACKING THAT ANTIGEN has produced antibodies in response to a stimulus. This sensitization can be purposeful, as in the case of human Rh negative volunteers who allow themselves to be injected with Rh positive blood with the aim of selling their serum to commercial companies as a source of Rh antisera. In these cases, the circumstances and conditions are carefully controlled. Unfortunately, the same result -- sensitization -- occurs accidently. As we have seen, an Rh negative person receiving Rh positive blood will usually build up a considerable concentration of anti-Rh antibodies in his blood. If he is mistakenly given a second transfusion of Rh positive blood at a later date, he may have a severe hemolytic transfusion reaction.

Sensitization by transfusion, under ordinary circumstances, is the result of careless and sloppy work on the part of the laboratory technician! However, a person may be sensitized and build up a high titer of anti-Rh antibodies without ever having a transfusion. An Rh negative woman married to an Rh positive man may, as we have seen, have an Rh positive infant. On occasion, the red blood cells of the fetus cross the placental barrier to reach the mother's bloodstream. Lacking the Rh antigen, she produces antibodies, anti-Rh. In the mother no reaction can take place since she has no antigen on her cells to combine with her circulating antibody. However, these antibodies may cross the placental membranes in the opposite direction, entering the fetus' bloodstream. Here the antibodies combine with the cells of the fetus and cause the condition known as hemolytic disease of the newborn (HDN).

Once the antibodies have been detected in the mother's serum, little can be done to protect her unborn child, except to alert the obstetrician, pediatrician, and blood bank to the possibility of an affected infant. For that particular family, though, the determination of the father's Rh genotype can be important in the estimation of the chance of future pregnancies in unaffected infants. Since the mother is Rh negative, she must be homozygous d or dd. The father, since his cells react with anti-Rh_o (D) antiserum, must have at least one gene for the Rh antigen. If he inherited an Rh_o (D) gene from each of his parents, he is homozygous for antigen Rh_o (D), will be genotype DD, and all his children will be Rh positive with a chance of being affected by HDN. If one of his parents passed on to him the gene for d, he is then heterozygous for the Rh factor, or genotype Dd, and statistically 50 percent of his children will lack the Rh_o (D) antigen. These children will be unaffected. By the use of a combination of family studies (the husband's parents and his other children), charts prepared from statistical studies and reaction of the husband's cells with available antisera, his Rh genotype can be estimated. Until someone discovers anti-d antiserum, the determination of genotypes is an educated guess.

We have discussed, at some length, the production of anti-Rh antibodies as a result of sensitization of an Rh negative individual by introduction of the antigen into the circulatory system. The antibodies produced may be "complete" or saline-active. They may also be "incomplete;" that is they will react with Rh positive cells only when a high concentration of protein is present, or in the antihuman globulin test. In Rh_o (D) incompatible pregnancies, at least, the usual pattern is for the saline-active antibody to appear first. This gradually decreases in concentration while the albumin active antibody simultaneously appears and gradually increases in concentration. That both kinds of antibody are produced is evidenced by the fact that both are commercially available, the companies having obtained them from Rh negative humans who have been sensitized (accidently or on a volunteer basis).

What we have discussed has been fairly straightforward. May we assume that a person is Rh negative because his cells do not agglutinate in the presence of anti-Rh_o (D) antiserum? No, this cannot be safely assumed. The Rh_o (D) antigen occasionally manifests itself as a variant which is designated by the term Rh_o (D^u). Ordinarily, this antigen is inherited in the same manner as the other antigens in the Rh system. The Rh_o variant (D^u) gives weak or, more often, negative responses with anti-Rh_o (D). The reaction is often stronger in a high protein medium. However, the method of choice for detection of this antigen is the antihuman globulin test since it is the most sensitive. Information concerning the presence or absence of the Rh_o variant (D^u) on apparently Rh negative cells can be of considerable clinical importance because Rh_o (D^u) positive blood given to an Rh negative patient may result in that patient's production of D antibody against Rh_o (D^u).

The procedure for determination of the presence or absence of the Rh factor on the cell is very much like that used for other blood group antigens. It is based on the bringing together (under controlled conditions) unknown cells with a specific antibody to see if an observable reaction results. Since the specific antibody in Rh determinations may be "complete" or "incomplete," great care must be taken to follow the manufacturer's directions precisely in setting up the test conditions. All Rh antisera sold commercially must meet National Institutes of Health (NIH) standards. The titer (concentration of antibodies in the serum) must be such that the serum diluted 1:32 will give visible agglutination of cells which contain the Rh_o antigen. Since the anti-Rh_o antibody reacts most strongly at body temperature, the use of a view box is advisable. The temperature of the glass plate on the view box should be 45° to 50° C so that the slide placed on it will have the optimum temperature of 37° to 40° C when the serum-cell mixture is read for agglutination.

Other equipment necessary in the slide method Rh_o (D) determination is a clean glass slide, applicator sticks, and an indelible marker.

Begin by first labeling a slide with the patient's identification. On the slide place two drops of anti-D. Using two applicator sticks, place one drop of the patient's blood next to the drops of anti-D. Then, with a clean applicator stick, mix the anti-D and blood until most of the slide is covered. Place the slide on the view box and tilt it for two minutes, then read for agglutination. (NOTE: Read at 2 minutes; any result after two minutes is rendered invalid due to drying of slide.)

Any agglutination on the slide indicates a positive test result, and the individual is termed "Rh positive."

If, on the other hand, no agglutination is observed, this yields a negative test result. Further testing is indicated, i.e., proceed to perform the test tube determination.

Equipment necessary for this procedure is 2 test tubes, an indelible marker, applicator sticks, serofuge, and bovine albumin (to be used as a control). As with the slide method, anti-D is used as a reagent.

Begin by labeling 2 tubes with the patient's identification. Label one tube "D" and the other "con." Into the tube marked "con" add 2 drops of bovine albumin and into the "D" tube place 2 drops of anti-D. Dip one applicator stick 1/8" to 3/16" into the patient's cells and transfer this to the "con" tube. Repeat this procedure with a clean applicator stick, transferring cells to test tube "D." After insuring that contents of both tubes are well mixed, serofuge for 15 seconds and read for agglutination.

Remember: the control tube must always be negative (no agglutination) if the results are to be considered valid. If the control is positive, we must ascertain the reason for agglutination.

If the sample tube shows agglutination, the blood bank technician reports the individual being tested as "Rh positive." On the other hand, no agglutination (negative test result) is not a guarantee that the individual is a true "Rh negative" since he may be "D^u positive." Further testing is again indicated.

The D procedure is simply a continuation of testing up to this point. Place the "con" and "D" tubes into a hot block (37° C) and incubate for a minimum of 15 minutes, a maximum of 60 minutes (depending on manufacturer's instructions). At the end of the incubation period, wash the cells in both tubes 4 times with saline.

Following the last wash, decant the clear supernatant. Add 2 drops of antihuman globulin to each tube and resuspend the cell button. Serofuge for 15 seconds and read for agglutination.

The "control" tube must remain negative (no agglutination). If the sample tube shows agglutination (positive test result), the individual is D^u positive, and is therefore reported as "Rh positive." However, if the patient's cells still demonstrate no agglutination (negative test result), the patient is a true "Rh negative" and is reported as such.

Though the determination of the Rh phenotype is not particularly complicated, there are some common sources of error of which we must be aware in the performance of the test. One of these is rouleaux formation (the "stack of coins" appearance of the cells) which grossly may appear to be agglutination; another is drying of the serum-cell mixture (particularly on slides). Both of these may be mistaken for agglutination (hence, a positive result) by the inexperienced technician. There are other sources of error as well. The antiserum may be contaminated, either by bacteria or by inadvertently mixing it with some other antiserum, so that specificity is lost. Improperly cleansed glassware may have in or on it some serum from a previous test, with the same result. The use of an excessive amount of anticoagulant can chemically coat the individual cells to the extent that agglutination with specific antibody is prevented. The technician may have too many cells for the amount of antiserum for the number of cells being used. AGAIN, WE MUST EMPHASIZE THE IMPORTANCE OF FOLLOWING DIRECTIONS EXACTLY; FAILURE TO DO SO AT ANY STEP OF THE PROCEDURE MAY GIVE INACCURATE RESULTS!

In your assignments as laboratory technicians you will be performing countless Rh_o determinations. Never let this task become routine! In this, as in any other laboratory procedure, your understanding of the principles and meticulous attention to details will enable you to report accurate and timely results. These results -- the end point of your work -- will determine the success or failure of your mission.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-105-1
LABORATORY SCIENCES DIVISION 014
CLINICAL MICROSCOPY BRANCH

STUDY ASSIGNMENT

Rh Typing and D^u Determination

The following questions are to be answered after class and returned to your instructor at the time specified. Your classroom notes and any available reference material may be used.

1. Commercial companies ordinarily obtain their Rh typing serum from ______________________.

2. A person's Rh type (positive or negative) is dependent on the presence or absence of _______________ antigen on his cells.

3. Below are six Rh blood group antigens as they have been designated by Doctor Alexander S. Wiener. In the space opposite each give the Fisher-Race designation.

Wiener	Fisher-Race
Rh_o	__________
rh'	__________
hr''	__________
rh''	__________
hr'	__________
Hr_o (theoretical)	__________

4. Why can we not prove the existance of the antigen Hr_o (d)?

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*This mimeo supersedes M 08-280-105-1, 061.

5. Define "phenotype."

6. Define "genotype."

7. A person with genotype CDe/cde is phenotype Rh______________.
Why?

8. A person with genotype Cde/cde is phenotype Rh______________.
Why?

9. Can two Rh negative parents have an Rh positive child?
Explain your answer.

10. Can two Rh positive parents have an Rh negative child?
Explain your answer.

11. An Rh positive person can be homozygous or heterozygous for the D antigen, but an Rh negative person must be homozygous (d/d). Why?

12. Define "sensitization."

13. The finding of anti-A and anti-B in human plasma or serum is perfectly normal. Is anti-Rh_o (D) normally found in humans?

____

14. A person who lacks the Rh_o (D) antigen may be sensitized to that antigen or purpose (commercial donors); however, Rh negative individuals may be sensitized accidentally as a result of __________ and/or __________.

15. Considering only the Rh_o (D) factor, can we safely transfuse an Rh positive person with Rh negative blood? __________ Explain your answer.

16. Considering only the Rh_o (D) factor, can we safely transfuse an Rh negative person with Rh positive blood? __________. Explain your answer.

17. An Rh negative patient, receiving his first transfusion, is accidentally given Rh positive blood. Will he suffer a transfusion reaction from this unit of blood? Explain your answer.

18. Explain the possible clinical importance of the presence of anti-Rh_o (CD) antibodies in the plasma of an Rh negative woman.

19. Can you always report a blood specimen as Rh negative because the cells fail to react with anti-Rh$_o$ (D)? ________________.
If not, what other determination(s) must be done? __________.
If further work gives positive results, how will your completed report read? ____________________.

20. Anti-Rh$_o$ (D) antibodies may occur as "complete," or ____________ reactive, but are more usually found as ____________ antibodies requiring a high protein concentration to react with the corresponding antigens.

21. Those antibodies requiring the high protein concentrations react best at a temperature of _______ ° C.

22. The glass top of the viewbox used in Rh determinations reaches a temperature of ______ ° C. to ______ ° C. so that the serum-cell mixture on the slide will be at optimum reaction temperature.

23. When using the saline-active anti-Rh serum the recommended concentration of red cells is ________ percent.

24. Cell concentration of ______ to ______ percent is recommended when determining Rh type by the slide method.

25. The most common cause of inaccuracy in determining Rh type is failure to ____.

26. Certain Rh agglutinogens Rh$_o$ (D) react with some but not all specific antisera; these agglutinogens are classified as _________________ of Rh$_o$ (D).

27. The presence of Rh$_o$ (D^u) agglutinogens is detected by incubating red cells with anti-D followed by the ___________ technique.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
CLINICAL MICROSCOPY BRANCH

*LP 08-280-104
048

Antihuman Globulin Tests (1 hour)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic)

PLACE: Classroom

REFERENCES:

TM 8-227-3

STUDY ASSIGNMENT: None.

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: Not applicable

PERSONNEL: One instructor (MOS 92B2H, 92B3H, or 92B4H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, eraser, overhead projector, transparencies.

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

I. INTRODUCTION (5 min)

A. Opening Statement: In the field of immunohematology, probably the most important single finding in the past 30 years was the "rediscovery" in 1945 of the antiglobulin test by Coombs, Mourant, and Race. It is of interest that, as an indication of the future significance of the test, it not only was successful in detecting the presence of weak and immune Rh antibodies, but also helped in the detection of many new antigen-antibody systems.

B. Objectives

1. State the principles on which the direct and indirect antihuman globulin tests are based.

2. List three applications of the direct and indirect antihuman globulin tests.

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*This lesson plan supersedes LP 08-280-104, 075.

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C. Class Procedure and Lesson Tie-in

This hour on the antihuman globulin test is part of a total of 64 hours of instruction, practice, and examination on theories, principles, and performance of routine pre-transfusion tests and the standard three-phase compatibility test.

FIRST HOUR

II. EXPLANATION (35 min)

A. Types of Antibodies

1. Complete (saline reactive)

2. Incomplete

a. Protein reactive

b. Coombs (antihuman globulin) reactive

B. Antihuman Globulin

1. Source: rabbits

2. Action: "bridges" between incomplete antibodies

3. Function: antibody to detect antibodies

C. Antihuman Globulin Determination

1. Direct method

a. Principle: detects in vivo coating of red cells

b. Applications

(1) Diagnosis of hemolytic disease of the newborn (HDN)

(2) Investigation of transfusion reactions

(3) Investigation of acquired hemolytic anemia (AHA)

c. Equipment

(1) Small test tubes (10 x 75 mm)

(2) Kimwipes

(3) Saline (0.90%)

(4) Serofuge

d. Reagent: Antihuman globulin

e. Controls

(1) Checks antihuman globulin

(2) Checks washing technique

f. Technique

(1) Label test tubes with proper patient identification.

(2) Add two drops of patient's 5% saline cell suspension.

(3) Wash four times with saline.

NOTE: At the end of the fourth wash pour off the saline and blot the lip of the tube.

(4) Add two drops antihuman globulin and resuspend cell button.

(5) Serofuge 15 seconds and read for agglutination; record results.

g. Interpretation of results

(1) Agglutination

(a) Positive test result

(b) In vivo coating of red cells

(2) No agglutination

(a) Negative test result

(b) No in vivo coating of red cells

2. Indirect method

a. Principle: detects circulating antibodies

b. Applications

(1) Detection of irregular antibodies (antibody screen)

(2) Investigation of transfusion reactions

(3) Modified for detection of D^u antigen

(4) Compatibility testing

c. Equipment

(1) Small test tubes (10 x 75 mm)

(2) Kimwipes

(3) Saline (0.90%)

(4) Serofuge

(5) Heating block , 37° C

d. Reagents

(1) Bovine albumin -- 22%

(2) Antihuman globulin

(3) Pooled group "O" screening cells

QUESTION: Why do we use group "O" screening cells when performing the indirect Coombs procedure?

ANSWER: We are looking for antibodies other than ABO antibodies. Group "O" cells do nothave the A or B antigens; therefore, anit-A and/or anti-B will not react with the screening cells.

e. Controls

(1) Checks antihuman globulin

(2) Checks washing technique

(3) Checks incubation time

f. Technique: three-phase

(1) Label test tube with proper patient identification.

(2) Add two to three drops patient's serum.

(3) Add two drops reagent cell suspension.

(4) Serofuge 15 seconds, read, and record results.

(5) Add two drops of 22% bovine albumin.

(6) Serofuge 15 seconds, read, and record results.

(7) Incubate at 37° C for 15 minutes.

(8) Serofuge, read, and record results.

(9) Wash four times with saline.

NOTE: At the end of the fourth wash, pour off the saline and blot the lip of the tube.

(10) Add two drops antihuman globulin and resuspend cell button.

(11) Serofuge, read, and record results.

QUESTION: Why do we use patient's serum rather than his or her cells?

ANSWER: We are attempting to detect antibodies (which are in the serum) rather than antigens which are on the surface of the cells.

g. Interpretation of results

(1) Agglutination

  (a) Positive test result

  (b) Antibody present in serum

(2) No agglutination

  (a) Negative test result

  (b) No antibody detected

NOTE: A negative result does not mean that there are no antibodies present, it only means that none were detected using these particular screening cells.

D. Sources of Error

1. Failure to follow manufacturer's directions

2. Failure to run controls

3. Specific antigen sites absent in reagent cells (indirect method)

4. Inadequate washing

5. Insufficient incubation (indirect method)

E. Questions from Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Types of antibodies

2. Antihuman globulin

3. Antihuman globulin determinations

4. Sources of error.

B. Closing Statement: A thorough understanding of the antihuman globulin test is of utmost importance since there may be situations when, instead of a test tube, you will almost literally hold a human life in your hands.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *IM 08-280-104
LABORATORY SCIENCES DIVISION 048
CLINICAL MICROSCOPY BRANCH

Antihuman Globulin Tests

In the field of immunohematology, probably the most important single finding in the past 30 years was the "rediscovery" in 1945 of the antiglobulin test by Coombs, Mourant, and Race. It is of interest that, as an indication of the future significance of the test, it not only was successful in detecting the presence of weak and immune Rh antibodies, but also helped in the detection of many new antigen-antibody systems.

The antihuman globulin (AHG) test was first described and used by Moreschi in 1908. He utilized the principle of red cells coated with an immune antibody and the addition of an antihuman globulin to help him detect bacterial agglutination. Since no one realized the test's potential, it did not attain widespread use and was soon forgotten. It was not until 1945 that Coombs, Mourant, and Race "rediscovered" the old principle and put it into practical use in immunohematology.

You will remember that when an antigen (foreign protein) is introduced into a human or animal, the result usually is the production of an antibody specific for that antigen. The antibody produced may be large, 950 Å (Å = 10^{-10} meter) and when the corresponding antigen combines with the antibody the reaction between the two will cause visible agglutination of the cells. The antibody, then is termed "saline reactive" or "complete."

In cases where the antibody is small, it does not cause visible agglutination of the cells it coats. This antibody may be "protein reactive" (250 Å) or "Coombs reactive." In either case, a medium other than saline is employed to bring red cells having coated antigen sites into closer proximity, thus making agglutination visible.

To understand the principle of the antihuman globulin test, it should be understood that all immune antibodies are globulin in nature; animals develop antihuman globulin antibodies when injected with human serum or purified globulins. When these AHG antibodies are added to cells previously coated with immune antibodies, they (AHG antibodies) "bridge" the gap between the coated cells, causing visible agglutination. Antihuman globulin, then, may be considered an antibody to detect antibodies. An indicator of the results is visible agglutination or the lack of it.

Coombs defined the AHG tests, which he developed, as DIRECT and INDIRECT. The direct AHG procedure determines whether red cells have been coated in vivo (in the body) with incomplete antibodies. The direct Coombs test is most commonly used in diagnosis of hemolytic disease of the newborn (HDN). Cord blood cells of an infant are tested to determine if the infant's erythrocytes have been coated from the mother's serum.

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*This Instructor's Manuscript supersedes IM 08-280-104, 075.

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The direct AHG test is also used in investigation of transfusion reactions. It is utilized since all the antibody may be absorbed on the cells so that none can be detected in the serum when the cells are removed. In this case, care must be taken in reading the direct AHG test, as a small amount of antibodies will result in microscopic mixed field agglutination and may be interpreted incorrectly as negative by the inexperienced technician.

The direct AHG test is also employed in the investigation of acquired hemolyti anemia (AHA), where an individual develops antibodies which coat his own cells, causing hemolysis of the coated cells within his body. Caution must be exercised in the interpretation of this test, as the direct AHG test is not always positive in these cases, and a negative test should not be considered to rule out acquired hemolytic anemia.

Equipment necessary for the direct procedure includes small test tubes (10 x 75 mm), Kimwipes, saline (0.90%), and a Serofuge while the reagent used is antihuman globulin. As in other laboratory procedures, an adequate control is a must if the results are to be considered valid. This control is used to check the reactivity of the AHG sera and to make sure your washing technique is correct.

Begin by labeling a test tube with the proper patient identification. Place two drops of a five percent saline suspension of the patient's red cells into the tube. Wash four times with saline. It may be necessary to wash cord bloods as many as 12 times if Wharton's jelly is present. After the last wash, decant the supernatant and blot the lip of the tube dry; add drops of AHG, and resuspend the cell button. Serofuge for 15 seconds and read for agglutination.

Agglutination, a positive test result, indicates an antibody is coating the patient's erythrocytes. No agglutination; i.e., no clumping, is a negative test result indicating no antibody coating the patient's erythrocytes.

The indirect AHG determination is a three-phase test used with pooled reagent Group "O" cells to detect incomplete antibodies in the serum of persons who have become sensitized to one or more blood group antigens. The test is commonly used in the study of immunization resulting from pregnancy, particularly when the woman is Rh negative. Another use of the indirect AHG test is in crossmatching. The test is of particular importance in checking the recipient's serum for antibodies. The determination may be used with known cells to identify antibodies in serum; by the same token, when serum of known antibody content is checked against unknown cells, the antigenic make up of the cells can be determined. This can be particularly important in the case of seemingly Rh negative donors who actually have on their cells the antigen, Rho variant (D^u).

Equipment for the indirect Coombs determination includes small test tubes (10 x 75 mm), Kimwipes, saline (0.90%), Serofuge, and a 37° C heating block. Reagents necessary are 22% bovine albumin, antihuman globulin, and pooled group "O" screening cells.

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As with the direct test, an adequate control is necessary. The control must be run to indicate reactivity of the AHG reagent with immune antibodies, to check incubation time, and washing techniques. The control should be treated exactly as the unknown.

Label a test tube with the proper patient identification. Place two to three drops of the patient's serum into the tube and add two drops of reagent cell suspension. Serofuge for 15 seconds and read for agglutination and/or hemolysis; add two drops of 22% bovine albumin. Serofuge for 20-30 seconds and read for agglutination. Incubate the tube at 37° C for a minimum of 15 minutes and a maximum of 60 minutes, depending on manufacturer's instructions. Following incubation, Serofuge for 20-30 seconds and read for agglutination, then wash at least four times with saline. After the last wash, decant the supernatant, blot, and add two drops of antihuman globulin. Resuspend the cell button, serofuge for 15 seconds, and read for agglutination.

Agglutination is a positive test result, indicating a circulating antibody present in the patient's serum. No clumping, on the other hand, is a negative test result which indicates no circulating antibody detected.

The most common source of error in both direct and indirect Coombs procedures is inadequate washing of cells. Failure to wash all excess protein from the cell suspension is a major source of false negative reactions due to neutralization of the Coombs serum by human protein.

In addition, the manufacturer's instructions must be adhered to rigidly. Never over-estimate your recall of procedures -- always read instructions, since they may vary from manufacturer to manufacturer. Reactivity of the sera, too, should be checked against appropriate controls. The importance of maintaining strict laboratory control over Coombs procedures cannot be overly emphasized.

A thorough understanding of the antihuman globulin test is of utmost importance since there may be situations when, instead of a test tube, you will almost literally hold a human life in your hands.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-106
LABORATORY SCIENCES DIVISION 075
CLINICAL MICROSCOPY BRANCH

Compatibility Testing (1 hour)

COURSES PRESENTED TO: 311-92B20 Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES:

TM 8-227-3

STUDY ASSIGNMENT: After class, study notes taken during class.

STUDENT UNIFORM AND EQUIPMENT: Not applicable.

TOOLS, EQUIPMENT, AND MATERIALS: M 08-280-106-1; -2 handed out as noted in LP.

PERSONNEL: One primary instructor (MOS 92B4H or 92B3H)

INSTRUCTIONAL AIDS: PA system with lapel microphone, chalk, chalkboard and eraser; SF 518 (one per student).

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Lecture

I. INTRODUCTION (5 min)

A. Opening Statement: You are holding two units of blood in your hands of the same ABO group and Rh type. Given in transfusion, one unit of blood will enable the patient to make a full recovery while the other unit may cause death. How are you to know which unit to give the intended recipient? The solution to this enigma is the compatibility test. In performing the crossmatch you have the power of decision and the corresponding responsibility.

NOTE: Hand out M 08-280-106-1

B. Objectives:

1. Identify the components of the major and minor sides of a crossmatch.

2. List the phases of compatability testing, to include necessary reagents for each phase.

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*This lesson plan supersedes LP 08-280-106, 074.

3. Recall the types of transfusion reactions.

4. Given the blood group and Rh type of both donor and recipient, predict possible crossmatch incompatabilities.

5. Recall the order of preference for selection of blood used for other than group specific transfusion.

C. Class Procedure and Lesson Tie-In:

1. One hour of instruction of a 64-hour block in immunohematology.

2. Practical exercise follows in accordance with individual needs.

3. Conferences when necessary to solve individual problems.

II. EXPLANATION (35 min)

A. Definition and Purpose of Compatibility Test

1. Pretransfusion compatibility testing

a. Compatibility

QUESTION: What does "compatibility" mean?

ANSWER: Existing together harmoniously.

b. Incompatibility

2. Detects errors

a. ABO cell and serum grouping

b. Labeling

c. Identification

NOTE: The crossmatch may not be relied upon to detect errors in Rh_o (D) typing.

B. Preparation for Compatibility Testing

NOTE: Issue SF Form 518, one per student.

1. Material for crossmatch
    a. Recipient's cells and serum
        (1) Fresh serum (less than two days old)
        (2) Complement (not present in plasma or aged serum)
    b. Donor's cells and serum (or plasma)
2. Patient (recipient) screening
    a. ABO grouping (cell and serum techniques)
    b. Rh_o (D) typing
    c. Antibody screening
3. Donor screening
    a. Retest ABO group
    b. Retest Rh_o (D) type
    c. Antibody screening

QUESTION: What is the most common source of error in the blood bank?

ANSWER: Human error!

4. Selection of donor blood
    a. ABO group and Rh_o (D) type specific
    b. Older units first

C. Compatibility Test

NOTE: Hand out M 08-280-106-2

1. Major side

a. Antigen (donor's cells)

b. Antibody (recipient's serum)

2. Minor side

a. Antigen (recipient's cells)

b. Antibody (donor's serum)

3. Three reaction phases

a. Saline

QUESTION: What errors should the saline phase of the crossmatch detect?

ANSWER: Errors in ABO grouping.

b. High protein

c. Antihuman globulin (Coomb's)

D. Three Phase Crossmatch

1. Principle

2. Equipment

3. Reagents

4. Procedure

a. Label 2 tubes: one for major crossmatch, and one for minor crossmatch

b. Place 2 drops of recipient's serum in major tube

c. Place 2 drops of donor's serum in minor tube

d. Add 2 drops of donor's cell suspension (4 - 6 percent) to major tube

e. Add 2 drops of recipient's cell suspension (4 - 6 percent) to minor tube

f. Serofuge for 15 seconds and read for agglutination and/or hemolysis (Saline Phase)

g. Resuspend cell button completely

h. Add 2 drops of 22 percent bovine albumin and mix

i. Serofuge for 15 seconds and read for agglutination and/or hemolysis (high protein phase, unincubated)

j. Resuspend cell button completely

k. Incubate at 37°C for 15 minutes

l. Serofuge for 15 seconds and read for agglutination and/or hemolysis (high protein phase, incubated)

m. Wash cell button completely

NOTE: The cell button must be washed until all free protein is removed from suspension. A minimum of four washes is recommended.

n. Add 2 drops of AHG (Coomb's) serum, and mix

o. Serofuge for 15 seconds and read for agglutination an/or hemolysis (antihuman globulin, or Coomb's phase)

5. Interpretation of Results

   a. Agglutination (or hemolysis) - incompatible

   b. No agglutination (or hemolysis) - compatible

E. Emergency Crossmatch Procedures

1. Urgent situations

   a. Uncrossmatched blood

   b. Physician must sign release

   c. Begin compatibility test procedures

   d. Selection of blood

      (1) Group and type specific

      (2) Group O, Rh_o (D) negative

      (3) Group O, Rh_o (D) positive

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2. Group AB recipient
    a. Order of selection (non-group specific units)
        (1) Group A (contains anti-B)
        (2) Group B (contains anti-A)
        (3) Group O (contains anti-A and anti-B)
    b. Antibody testing
        (1) No hemolysins (no hemolytic anti-A and/or anti-B)
        (2) Low titre (less than 1:50 titre for anti-A and/or anti-B agglutinins)
3. Non-group specific compatibility test
    a. Major side (must be negative)
    b. Minor side (should be positive)

F. Disposition of Blood and Specimens
1. Certification of crossmatch
2. SF Form 518 kept with unit of blood
3. Release of crossmatched blood
4. Seven day retention of crossmatch specimens
5. Fresh patient specimen (each day of transfusion)

G. Possible Transfusion Reactions
1. Hemolytic
2. Allergic
3. Febrile

H. Questions from Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Purpose of compatibility test

2. Preparation for compatibility test

3. Compatibility test

4. Three phase crossmatch

5. Emergency crossmatch procedure

6. Disposition of blood and specimens

7. Possible transfusion reactions

B. Closing Statement: The blood bank is engaged in the task of providing blood for transfusions. Your ability to perform accurate and rapid compatibility tests may literally mean the difference between life and death to the patient. An error at the Academy of Health Sciences will affect only your grade, but a mistake in the blood bank may have tragic consequences for the person who depends on you for help...the patient!

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *IM 08-280-106
075
LABORATORY SCIENCES DIVISION
CLINICAL MICROSCOPY BRANCH

Compatibility Testing

You are holding two units of blood in your hands of the same ABO group and Rh type. Given in transfusion, one unit of blood will enable the patient to make a full recovery while the other unit may cause death. How are you to know which unit to give the intended recipient? The solution to this enigma is the compatibility test. In performing the crossmatch you have the power of decision and the corresponding responsibility.

The compatibility test, or crossmatch, is actually a battery of laboratory procedures performed before transfusion to insure serologic compatibility between the proposed blood donor(s) and the recipient, and assure maximum therapeutic benefit for the recipient. Each crossmatch is a unique experiment in which the unknown serums and cells are tested in vitro for detection of antibodies. Any reaction is indicative of incompatibility; negative results are construed to indicate compatibility.

The crossmatch is used to detect antibodies in the recipient's serum specific for the donor's erythrocytic antigens, and to detect donor antibodies directed against the recipient's red cells. The crossmatch is especially helpful in detecting errors in ABO group determination, such as when non-group specific transfusion is unintentionally attempted. The compatibility test is used to minimize errors encountered in the labeling of the units of donor blood, and errors in identifying the donors and recipients.

It should be noted that the crossmatch may not be relied upon to prevent immunization of the recipient, since the foreign antigen has not yet stimulated the production of a specific antibody in the recipient. For example, if Rh_o (D) positive blood is crossmatched for an Rh_o (D) negative recipient, the compatibility test will be negative (unless the recipient has been previously sensitized to the Rh_o (D) antigen). Further, the crossmatch may not be relied upon to detect all procedural errors or insure normal survival of the donor's erythrocytes after transfusion.

Prior to compatibility testing, a fresh specimen of blood from the intended recipient must be obtained. Each unit of blood, or blood components, must be requested on a separate SF 518 (Blood Transfusion). This request must include the patient's full name, hospital number, ward, sex and social security number. Despite all of the technical and

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*This instructor's manuscript supersedes IM 08-280-106, 074.

procedural advances in immunohematology, the major cause of incompatible hemolytic transfusion accidents remains outside of the blood groups. Most of these reactions are due to errors in the labeling and handling of recipient samples, or failure to give properly crossmatched blood to the correct recipient. The individual collecting the recipient's blood must identify both the intended recipient and his blood clearly and unmistakably prior to completing the verification statement on the SF 518.

The recipient's blood specimen should be collected without anticoagulant, since complement, a labile component of serum necessary for some antigen-antibody reactions, may be inactivated by the action of the anticoagulant. Serum used for the compatibility test must be less than two days old to insure the presence of complement. Donor's cells and serum (or plasma) may be obtained from the pilot tubes or from the integral tubing segments attached to the unit of blood.

The blood group of the recipient is determined by performing both cell and serum grouping procedures. The Rh_o (D) type of the recipient is determined with the use of tube test Rh_o (D) typing techniques. If this test is negative, a D^u determination is performed to identify D^u recipients who may be transfused with Rh_o (D) positive blood. The serum of all recipients should be tested for irregular antibodies. Antibody screening may be done beforehand, or as a part of the compatibility test procedures.

The American Association of Blood Banks requires that all ABO grouping and Rh_o (D) typing procedures be repeated on each unit of blood when the crossmatching facility differs from the collecting facility. It is advisable to confirm the donor's ABO group and Rh_o (D) type each time the unit of donor blood is crossmatched, thereby not relying entirely on the container label. Under normal circumstances, the donor unit(s) of blood selected for crossmatching is specific for ABO group and Rh_o (D) type with that of the recipient. The donor blood should be free of atypical antibodies. Antibody screening is usually accomplished when the donor's blood is initially processed and labeled, but may be included as part of the compatibility test procedures.

Once preliminary testing has been completed and selection of appropriate donor blood has been made, the crossmatch can be initiated. The crossmatch is divided into two separate procedures: The major crossmatch (or side) consisting of the donor's cells and the recipient's serum, and the minor crossmatch (or side) utilizing the donor's serum and the recipient's cells. The major crossmatch demonstrates the presence of antibodies in the recipient's serum for which the donor's erythrocytes possess specific corresponding antigens which would cause destruction of the donor's cells as they are introduced into the recipient's circulatory system. The primary purpose of transfusion is to compensate for anemia in the patient by increasing the oxygen carrying capacity with an infusion of erythrocytes. The major crossmatch is so called since the donor's cells are infused into the recipient. The minor crossmatch demonstrates antibodies in the donor's serum which will react with antigens on the recipient's erythrocytes. The minor crossmatch is considered less important in that the effects of donor antibodies are diluted by the recipient's circulatory system to a point where there is usually no severe reaction should a specific antibody be infused, either accidentally or on purpose. The minor crossmatch is routinely performed in military blood banks, but is not specifically required by the American Association of Blood Banks if the donor's serum has been adequately tested and found to be free of irregular antibodies.

You know, from your previous knowledge, that a visable reaction for a naturally occurring antibody (i.e., ABO system) occurs under different conditions than those for an immune antibody (i.e., Rh-Hr system). The three phase crossmatch has been designed to incorporate as many temperatures and media as practically possible, thus detecting both natural and immune antibodies. The saline phase crossmatch reacted at room temperature (20°C) will detect most saline reactive antibodies, such as those of the ABO system. The high protein phase crossmatch reacted at body temperature (37°C) will detect many immune antibodies. The antihuman globulin (Coomb's) phase crossmatch is performed to detect antibodies not previously demonstrated by either saline or high protein techniques.

The procedure for setting up and performing the two tube, three phase crossmatch is as follows:

1. Label two (2) test tubes (12 x 75 mm) with the recipient's name and the donor's identification number; one labeled for the major crossmatch and the second labeled for the minor crossmatch.

2. Place two drops of the recipient's serum into the tube labeled for the major crossmatch.

3. Place two (2) drops of the donor's serum into the tube labeled for the minor crossmatch.

4. Add two (2) drops of the donor's washed cell suspension (4 to 6 percent in normal saline) to the tube labeled for the major crossmatch.

The cell suspensions for use in the compatibility test procedures should be prepared by washing the cell suspensions until they are free of extraneous protein; usually this will require at least four washings. Thorough washing will remove surrounding serum (or plasma) and hemolized erythrocyte stroma and fibrin, thus exposing the antigen sites for reaction with any antibody present in the crossmatch medium.

5. Add two (2) drops of the recipient's washed cell suspension (4 to 6 percent in normal saline) to the tube labeled for the minor crossmatch.

6. Place both tubes in the Serofuge and centrifuge for fifteen (15) seconds. Read for agglutination and/or hemolysis. This is the saline phase of the crossmatch procedure. Most naturally occurring antibodies will be demonstrated at this point.

7. Resuspend the cell button completely with moderate agitation until all cells are in suspension.

8. Add two (2) drops of 22 percent bovine albumin to each tube and mix completely by gentle agitation.

9. Place both tubes in the Serofuge and centrifuge for fifteen (15) seconds. Read for agglutination and/or hemolysis. Many immune antibodies may be demonstrated at this point in the high protein phase of the crossmatch prior to incubation.

10. Resuspend the cell buttons completely with moderate agitation until all cells are in suspension.

11. Place both the major crossmatch and the minor crossmatch tubes in the incubator at 37°C for at least fifteen (15) minutes. This incubation period allows any antibody prasent to coat corresponding antigen sites so that a visible reaction may be demonstrated.

12. At the completion of the incubation period the crossmatch tubes are placed in the Serofuge and centrifuged for fifteen (15) seconds and read for agglutination and/or hemolysis. Immune antibodies which reacted with the antigen sites during the incubation period may be demonstrated at this point of the high protein phase of the crossmatch.

13. Wash the cell button in both crossmatch tubes until free of extraneous protein. At least four (4) washes with normal saline are recommended to remove all traces of unbound protein. Thorough washing of the cells is vital, since unbound human protein neutralizes the antihuman globulin, preventing a visible reaction. (Note: One volume of human protein will neutralize 4,000 volumes of antihuman globulin.)

14. Add two (2) drops of antihuman globulin (Coomb's serum) to the washed cell in each tube and mix completley by agitation.

15. Place both tubes in the Serofuge and centrifuge for fifteen (15) seconds. Read for agglutination and/or hemolysis. Almost all antibodies react in this antihuman globulin phase of the crossmatch, and it is, therefore, an essential part of every antibody screening and compatibility testing procedure.

Interpretation of results is an important as the proper performance of each step in the crossmatch procedure. Agglutination, hemolysis or both in any phase of the compatibility test renders that crossmatch incompatible. If all phases of the crossmatch are negative, on both the major side and the minor side, the blood is considered compatible. Test results are reported as "compatible" or "incompatible."

In most emergency situations, there is adequate time to perform a suitable compatibility test prior to issuing blood for transfusion. Nevertheless, each blood bank must be prepared to initiate emergency procedures to meet urgent situations. In an emergency, the patient's physician must weigh the dangers of infusing uncrossmatched blood against the risk to the patient of waiting for a proper and complete crossmatch. If the physician decides the situation warrants the use of uncrossmatched blood, this may justifiably be done. He must accept the responsibility for any reaction that would have been detected by the compatibility test, and he must indicate his acceptance in writing.

When blood is required in an emergency situation, give the uncrossmatched blood to the physician when he demands it. Have the physician sign a release at the time it is issued. Then begin the routine compatibility test procedure to insure serologic compatibility.

When sufficient time is not available to complete routine crossmatch procedures, action must be taken to provide necessary blood as rapidly as possible. If there is insufficient time for complete crossmatch procedures, there may be time to determine the recipient's ABO group and Rh_o (d) type. In this circumstance, group and type specific blood may be issued without crossmatch.

If time does not permit blood grouping of the patient, group O, Rh_o (D) negative blood (negative for hemolytic anti-A and anti-B) should be utilized to the extent of the blood bank's ability. In acute emergencies, the supply of group O, Rh_o (D) negative blood may become exhausted. Although it is less suitable, and may possibly cause sensitization (or a reaction in previously sensitized patients) in some Rh_o negative recipients, Group O Rh_o (D) positive blood (again free of hemolytic anti-A and anti-B) may be used if group O Rh_o (D) negative blood is not available.

If a limited amount of time (ten to fifteen minutes) is available, determine the recipient's group and type, and set up routine procedures. Emergency procedures may include a drastically reduced incubation prior to conversion to the antihuman globulin test, and/or simply an immediate spin prior to reading the major and minor crossmatches. These emergency procedures must be documented as acceptable techniques in the blood bank's standard operating procedures. Even though the blood is issued before the crossmatch is completed, routine crossmatch procedures must be completed and recorded. Completed test procedures may show an incompatibility not previously detected while there is still a chance of discontinuing the transfusion.

In many instances, group AB blood may not be available for transfusion to an AB recipient, due either to depletion of normal blood reserves or some urgent situation. Selection of non-group specific blood is based on several considerations, including the availability of the different ABO groups. It is usually considered preferable to administer either group A blood (containing anti-B) or group B blood (containing anti-A) to the group AB recipient since either group contains only one ABO agglutinin capable of reacting with the recipient's receptor sites. On occasion it may be necessary to use group O blood, although this is less desirable, since group O blood contains both anti-A and anti-B capable of agglutinating the recipient's native cells.

If the group AB recipient has received either group A or group B blood, his plasma will contain anti-A or anti-B. The decision to change to yet another blood group should be based on consideration of the antibodies concerned. (E.g., it would not be safe to change from group A blood to group B blood, since the recipient would then have both antigen A and anti-A, and antigen B and anti-B in addition to his native cells with A and B antigen sites.) In such circumstances, group O blood should be administered as long as either anti-A or anti-B remain detectable in the recipient's serum.

Blood which is to be used for non-group specific transfusion must be negative for hemolysins when tested for the presence of hemolytic anti-A and/or anti-B; and must have a titre of less than 1:50 when tested for naturally occurring anti-A and/or anti-B agglutinins. Preferably, non-group specific blood should be infused as packed cells with the plasma extracted, thus eliminating the introduction of antibodies capable of reacting with the recipient's native erythrocytes. Administration of hemolytic ABO antibodies will result in hemolysis of the recipient's erythrocytes in vivo, thus negating the desired effects of transfusion.

Whenever a patient is crossmatched with ABO heterospecific (non-group specific) blood the compatibility test procedure will normally indicate serologic incompatibility. The major crossmatch must always be negative to avoid infusing erythrocytes which would be destroyed by the patient's native antibodies as the cells enter his circulatory system. A major crossmatch reaction demonstrates the presence of an antigen for which a specific corresponding antibody is present in the patient's serum (e.g., transfusion of a group O patient with group A blood would result in the administration of antigen A to an individual with anti-A).

The minor crossmatch of a non-group specific compatibility test will invariably be positive, indicating the presence of an antibody in the donor's plasma specific for an antigen on the recipient's erythrocytes. However, non-group specific blood may usually be infused with a degree of safety; this is due to the relatively small volume of antibody introduced, compared with the much larger number of receptor sites available for reaction in the recipient. In effect, the antibodies are diluted by the recipient's circulatory system to the point where there is usually little or no clinically observable reaction, if the donor's blood is free of hemolytic ABO antibodies. (It should be noted that if heterospecific blood is administered as packed red cells no antibody will be infused for reaction with the recipient's erythrocytes.)

Once all of the compatibility test procedures are completed and appropriate log entries have been made, the crossmatch must be certified by the laboratory officer. This is the final point at which most possible errors may be detected and corrected prior to the release of the unit(s) of blood. The certifying officer will verify the apparent compatibility of the crossmatch and insure that all log entries and paperwork have been completed without error. Certification by the laboratory officer minimizes clerical and technical errors and assures maximum patient safety when the unit(s) of blood is transfused.

Each unit of blood must be labeled with the recipient's name, date of crossmatch, hospital number and the signature of the technician performing the crossmatch, in such a manner that it will not become separated from the unit when the blood is released for infusion. The SF 518 must accompany each unit of blood when it is released by the blood bank. The transfusion record portion of the SF 518 must be completed at the time the blood is administered, and one completed copy returned to the blood bank, together with the empty blood bag.

Before releasing crossmatched blood, the expiration date of each unit must be checked and the unit inspected for hemolysis or abnormal appearance. All information relating to the identity of both the unit of blood and the recipient must be compared with the unit of blood, the transfusion log book, the transfusion request and the label attached to the unit of blood. The release of blood for transfusion may be expedited by using a check list to insure that all of the necessary controls and inspections have been completed on each unit of blood prior to release. Final identification of the recipient and the unit of blood is the responsibility of the individual who actually starts the infusion.

Both the donor pilot tubes and the patient's blood specimen must be retained and stored at 4 to 6°C for a period of at least seven days after the completion of the transfusion. Once the transfusion is completed, the empty container must be returned to the blood bank and retained in the refrigerator for at least 24 hours. This retention of all specimens is vital to conduct a transfusion reaction investigation should the recipient suffer any adverse effects during or after the transfusion.

If a patient receives multiple unit transfusions over an extended period of time he will require special attention. A fresh specimen must be drawn each day of transfusion for crossmatch and antibody screening procedures. Since the patient is being exposed to foreign blood group antigens, it is possible that he will be stimulated to produce an antibody.

Blood transfusion is expected to benefit the recipient without causing adverse effects. Any unfavorable event in the recipient resulting from a transfusion is considered to be a transfusion reaction. The most dangerous transfusion reaction in the patient is a hemolytic transfusion reaction, so called because of massive in vivo hemolysis which may be immediately apparent or delayed up to several days. Hemolytic transfusion reactions are most often due to clerical error or error in identification, and may usually be prevented if positive identification is maintained on all specimens at all times. Allergic transfusion reactions are the most common adverse reaction to transfusion of blood; they are seldom severe and are probably due to the patient's response to allergens in normal blood. Allergic reactions are characterized by the presence of hives and itching (urticaria) or a diffuse rash, and respond to anti allergic medication. Febrile reactions may occur if bacterial pyrogens are introduced into the patient's system. Febrile transfusion reactions are characterized by chills and fever, and will usually respond readily to treatment with antipyretic drugs.

The blood bank is engaged in the task of providing blood for lifesaving transfusions. Your ability to perform accurate and rapid compatibility tests may literally mean the difference between life and death to the patient. An error at the Academy of Health Sciences will affect only your grade, but a mistake in the blood bank may have tragic consequences for the person who depends on you for help. . . the patient.

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SSG Andre Wynohrad

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-106
PATHOLOGY AND LABORATORY SCIENCES DIVISION 014
HEMATOLOGY BRANCH

Compatibility Testing

The following questions are to be answered after class and returned to your instructor at the time specified. Your classroom notes and any available reference material may be used.

1. Basically, in performing the compatibility test you are mixing the blood of the recipient with that of the intended donor outside the body to see if they are compatible. On the major side of the test, donor ______________ are mixed with recipient ______________; on the minor side, donor ______________ is mixed with recipient ______________.

2. The compatibility test is performed in three phases. These are the ______________, the ______________, and the ______________ phases.

3. What factor or factors could be detected in each of the three phases?

a. ______________________________

b. ______________________________

c. ______________________________

4. Besides agglutination, another type of reaction which could demonstrate an antigen-antibody reaction in the compatibility test is ______________.

5. Group O blood given to a Group A recipient would show a reaction in the ______________ side of the compatibility test. Why?

6. Group A blood given to a Group AB recipient would show a reaction in the ______________ side of the compatibility test. Why?

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*This mimeo supersedes M 08-280-106, 014.

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M 08-280-106
014

7. While you are on night call in the Blood Bank you receive an emergency request of 10 units of blood for an AB recipient, and find that you have only six units of Group AB blood. According to the American Association of Blood Banks (AABB), of the other three groups which may be given this recipient, what would be your first choice? ______________. Your second choice? ______________. Your third choice? ______________. What test should be performed on this donor blood before it is selected for use with this Group AB recipient?

8. After performing the compatibility test, all blood specimens on which the test was conducted must be saved in the refrigerator for a period of at least _________ days.

9. Three types of transfusion reaction may be seen in a transfused recipient. These are ______________, ______________, and ______________ in nature. Of these three, the ______________ reaction is most dangerous.

10. The transfusion reaction most commonly observed is ___________ in nature, and is characterized by the presence of a/an ____________ rash.

11. If a reaction is seen in any of the phases of the compatibility test, the donor and recipient are said to be ______________.

12. In the performance of the compatibility test, the recipient's serum is used, rather than plasma. Why?

13. Except in cases of emergencies, Rh positive blood is not given to an Rh negative recipient. Why not?

14. The form number of the tranfusion request is SF __________.

15. In your own words, explain the term "compatible".

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MSG Arnold I. Gorsch

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
CLINICAL MICROSCOPY BRANCH

*M 08-280-106-1
077

STUDY ASSIGNMENT

Compatibility

RECIPIENT	DONOR	AGGLUTINATION		SENSITIZATION	COMPATIBLE
		Major	Minor		
AB neg	AB pos	o	o	YES	NO
A neg	AB pos				
O pos	O neg				
B neg	AB pos				
B neg	B neg				
O pos	AB neg				
A pos	A pos				
AB pos	B neg				
AB pos	A pos				
AB neg	O neg				
O neg	O pos				
AB pos	AB pos				
A pos	A neg				
O pos	O pos				
B pos	AB neg				
A neg	A pos				
AB neg	AB pos				
B pos	B neg				
A neg	B pos				
AB pos	AB neg				
B neg	A pos				
A pos	B pos				
O neg	A pos				
B neg	O neg				
A neg	O neg				
A neg	AB neg				
B pos	O pos				
B neg	AB neg				
O pos	B pos				

*This mimeo supersedes M 08-280-106-1, 014.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
CLINICAL MICROSCOPY BRANCH

M 08-280-106-
104

Compatibility Testing Procedure

1. Label 2 tubes: one for the major crossmatch and one for the minor crossmatch.

2. Place 2 drops of recipient's serum in the major tube.

3. Place 2 drops of donor's serum in the minor tube.

4. Add 2 drops of donor's cell suspension (4 to 6 percent in normal saline) to the major tube.

5. Add 2 drops of recipient's cell suspension (4 to 6 percent in normal saline) to the minor tube.

6. Serofuge for 15 seconds and read for agglutination and/or hemolysis. (Saline Phase)

7. Resuspend the cell buttons completely.

8. Add 2 drops of 22 percent bovine albumin and mix.

9. Serofuge for 15 seconds and read for agglutination and/or hemolysis. (High Protein Phase)

10. Resuspend the cell buttons completely.

11. Incubate at 37°C for 15 minutes to 60 minutes.

12. Serofuge for 15 seconds and read for agglutination and/or hemolysis. (High Protein Phase, Incubated)

13. Wash cell button completely. (Note: At least 4 washes are recommended.)

14. Add 2 drops of AHG (Coomb's sera) and mix.

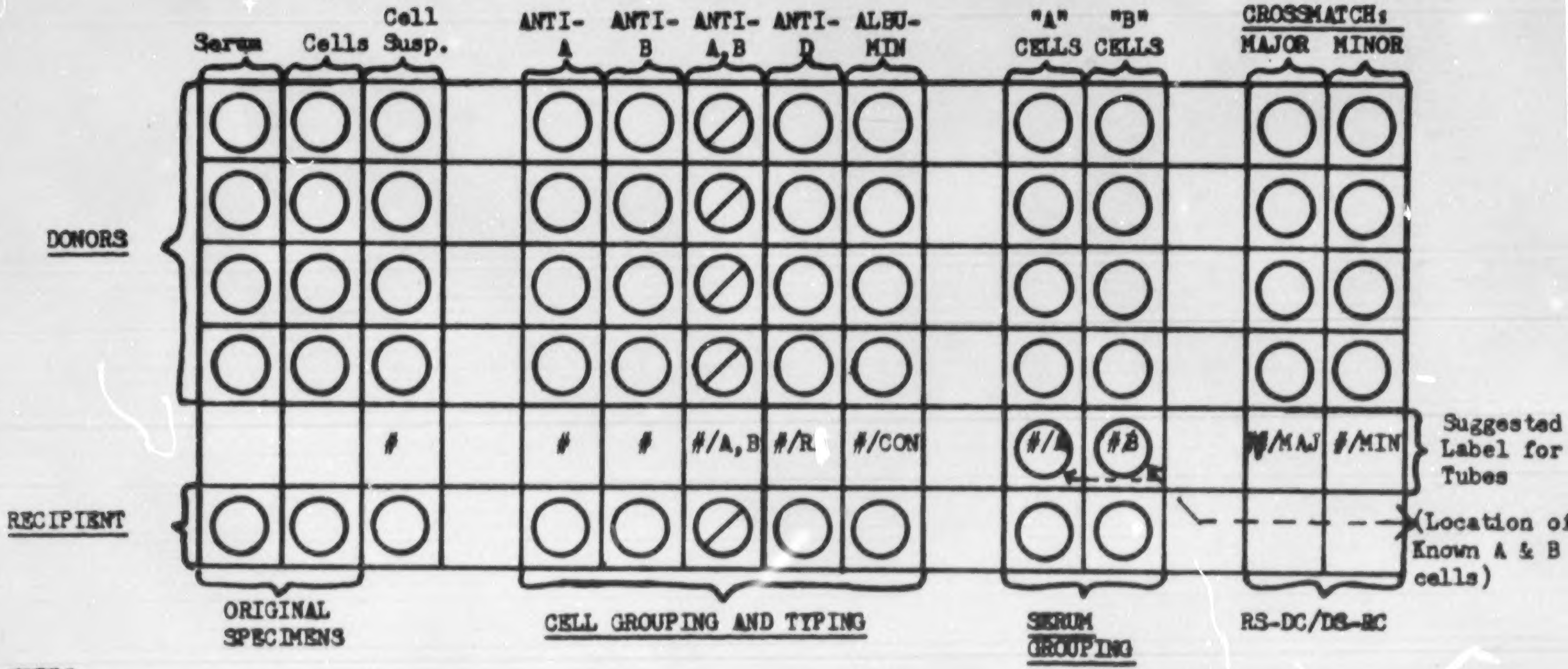
15. Serofuge for 15 seconds and read for agglutination and/or hemolysis. (Antihuman Globulin (Coomb's) Phase)

SSG Gary D. Jennings

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
CLINICAL MICROSCOPY BRANCH

*M 08-280-106-4
036

Organization of Rack for Crossmatch/Related Procedures


Serum
Cells
Cell Susp.
ANTI-A
ANTI-B
ANTI-A,B
ANTI-D
ALBU-MIN
"A" CELLS
"B" CELLS
CROSSMATCH:
MAJOR
MINOR
DONORS
RECIPIENT
#
#
#
#/A,B
#/R
#/CON
#/A
#B
#/MAJ
#/MIN
Suggested Label for Tubes
(Location of Known A & B cells)
ORIGINAL SPECIMENS
CELL GROUPING AND TYPING
SERUM GROUPING
RS-DC/DS-RC

NOTES:

1. ⊘ - Indicates that Anti-A,B should be employed only in the confirmation of Group "O" specimens.
2. Each test procedure calls for the use of 2 drops of reagent and 2 drops of the specimen to be tested.
3. RS-DC = 2 drops recipient's serum + 2 drops donor cell suspension. DS-RC = 2 drops donor serum + 2drops recipient cell suspension.

*This mimeo supersedes M 08-280-106-4, 014

SSG Stephen T. Kershaw

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY [illegible]-280-108
LABORATORY SCIENCES DIVISION (
CLINICAL MICROSCOPY BRANCH

Blood Donor Processing (3 hours)

COURSES PRESENTED TO: 311-92B20, Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES:

TM 8-227-3

STUDY ASSIGNMENT: Not applicable

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: None

PERSONNEL: One primary instructor (MOS 92B4H, 92B3H)

INSTRUCTIONAL AIDS: P.A. system with lapel microphone, chalk, chalkboard and eraser

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

I. INTRODUCTION (5 min)

A. Opening Statement: Human blood for transfusion is the most precious and at the same time, perhaps the most dangerous of any biological commodity routinely used in the practice of medicine today -- precious because it can be manufactured only by the human animal, and dangerous because once transfused into a patient it cannot be retrieved. It may be part of your duties as a laboratory technician to recruit blood donors, perform the phlebotomy to obtain the unit of blood, and perform the necessary processing to insure that the unit obtained is suitable for transfusion. Each step, from the initial donor interview

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*This lesson plan supersedes LP 08-280-108, 034.

through the processing to the final placement of that unit in the blood bank must be done correctly and with absolute accuracy. Truly, the operation of a blood bank must be a "zero defects" program!

B. Objectives

1. Discuss the medical and physical criteria used in the selection of blood donors.

2. Outline and explain the steps used in the procurement of blood from the donor.

3. List and explain the various techniques and safety devices used in the storage, issue, and transportation of blood.

4. Outline and explain the procedure used to detect the presence of atypical antibodies in donor blood.

5. Explain the necessary steps in the processing of whole blood prior to use.

C. Class Procedure and Lesson Tie-in

1. Three instructional hours of 64 hours on immunohematology.

2. Conferences when necessary to solve individual problems.

FIRST HOUR

II. EXPLANATION (85 min)

A. Blood Donor Reception

1. Comfortable surroundings

2. Friendly atmosphere

3. Professional actions

B. Blood Donor Records

NOTE: At this time issue DD Form 572 (Blood Donor Record Card), one per student.

1. Identifying information

a. Name

b. Grade

c. Social security account number

d. Sex

e. Organization

f. Address and telephone number (walking donor file)

2. Medical history

a. Protection of recipient

(1) Malaria (DD Form 572, item 10, 11, 28)

(2) Hepatitis (DD Form 572, item 14, 15, 16, 17)

(3) Immunizations (DD Form 572, item 19, 20, 22, 38, 40, 41, 42)

b. Protection of donor (DD Form 572, item 21, 29, 30, 31, 32, 33, 34, 35)

c. Other questions (DD Form 572, item 12, 13, 18, 27, 36, 37, 39)

3. Physical criteria

a. Weight: 110 pounds (minimum for full unit)

b. Temperature: Maximum oral temperature 99.6°F with no minimum

c. Pulse: 50-100 beats/minute

d. Blood pressure

(1) 90 to 180 mm mercury (systolic)

(2) Not to exceed 100 mm mercury (diastolic)

e. Arm inspection

f. Hemoglobin

(1) Male: 13.5 gm/100 ml

(2) Female: 12.5 gm/100 ml

g. Hematocrit

- (1) Male: 41 percent VPRC
- (2) Female: 38 percent VPRC

h. Age 17-65 (legal age; must not have reached his 66th birthday)

i. Frequency

- (1) Not less than 8 weeks apart
- (2) Not more than 5 times per year

C. Blood Donor Equipment

1. Containers

a. Plastic bag

- (1) Anticoagulant: 67.5 ml Acid Citrate Dextrose
    - (a) Anticoagulant
    - (b) Nutrient
    - (c) Buffer
- (2) Advantages
    - (a) Closed system
    - (b) Preservation of platelets
    - (c) Preservation of cells
    - (d) Resistance to breakage
    - (e) Storage space
- (3) Disadvantages
    - (a) Overfilling
    - (b) Sealing of tubing

b. Glass bottles

2. Phlebotomy equipment

D. Phlebotomy

 1. Selection of vein

 2. Cleaning of phlebotomy site (5-step surgical scrub)

  a. Surgical soap

   (1) Removal of grease and dirt

   (2) 1 1/2 inches in all directions with gauze

  b. Alcohol, 70 percent (with 10 percent acetone): removal of soap

  c. Tincture of iodine (3 percent in ETOH)

   (1) Spiral pattern

   (2) Allow to air dry

  d. Alcohol, 70 percent (to remove iodine)

  e. Apply sterile gauze pad (to cover puncture site)

E. Blood Collection

 1. Phlebotomy

  a. Needle (integral part of container tubing)

  b. Puncture

 2. Blood collection

  a. Identification procedures

  b. Quantity (450 ml + 67.5 ml ACD)

  c. Donor-technician relationship

  d. Mixing

  e. Pilot tubes

 3. Post-donation observation and care

  a. Protection of phlebotomy site

        - b. Possible reactions
        - c. Donor dismissal

QUESTION: What quantity of blood is routinely collected from a donor?

ANSWER: 450 ml

- F. Film (39 min) (see Annex A)
    - 1. Introduction (5 min)
        - a. Blood donation
        - b. Points to be observed
            - (1) Donor history
            - (2) Donor physical
            - (3) Phlebotomy
            - (4) Donor care
    - 2. Show AHS TV tape #12 (running time, 29 min)
    - 3. Discussion of tape or questions from students (5 min)
- G. Blood Storage, Issue and Transportation
    - 1. Length of storage (21 days with ACD)
    - 2. Refrigeration
        - a. Temperature
            - (1) 2° range between 1° and 6° C (33.8° and 42.8° F)
            - (2) Alarm: 1° to 6° C
    - 3. Inspection of stored blood
        - a. Daily visual inspection
            - (1) Hemolysis
            - (2) Clots
            - (3) Contamination

b. Rotation by age

NOTE: At this time issue SF 518 (Blood Transfusion) one per student.

4. Blood issue
    a. Rigid laboratory controls
    b. Accurate records
    c. Verification of identification (name and numbers)
        (1) Donor bag
        (2) Pilot tubes (or tube segments)
        (3) Transfusion request
        (4) Blood bank records

NOTE: At this time issue DD Form 573 (Shipping Inventory of Blood Collections) one per student.

5. Blood shipment
    a. Completion of DD 573 (Shipping Inventory)
    b. Shipping container
    c. Shipping temperature (1° to 10° C)
    d. Wet ice (dripping)
    e. Inspections, re-ice, etc.

QUESTION: For how long a period may whole blood be stored in the proper environment?

ANSWER: 21 days.

H. Blood components
1. Whole blood
2. Plasma
    a. Fresh frozen
    b. Plasma fractions

3. Blood cells

I. Precautions

1. Select personnel (trained and responsible)

2. Rigid laboratory controls

3. Accurate records

4. Suitable reagents

5. Proper storage of blood

6. Checklist for blood issue

J. Blood processing

1. Required laboratory testing

a. ABO

(1) Cell grouping

(2) Serum grouping

b. Rh typing (D^u testing)

c. Serological test for syphilis

d. Titer for Group O (anti-A and anti-B)

e. HAA (Hepatitis-Associated Australia Antigen)

f. Antibody screening (3 phase)

(1) Principle

(2) Equipment

(3) Reagents

(a) Screening cells, group O

(b) Antihuman globulin

(c) Bovine albumin

(4) Procedure

(a) Label tube with patient's identification

(b) Place 1-2 drops patient's serum in tube

(c) Add 1-2 drops screening cells and mix

(d) Serofuge 15 seconds and read for agglutination (saline)

(e) Resuspend cell button completely

(f) Add 2 drops bovine albumin

(g) Serofuge 15 seconds and read for agglutination (protein

(h) Resuspend cell button completely

(i) Incubate 15-60 minutes at 37° C.

(j) Serofuge 15 seconds and read for agglutination (protein

(k) Wash 3-6 times with saline

(l) Add 2 drops antihuman globulin and mix

(m) Serofuge 15 seconds and read for agglutination (AHG)

(5) Results

(a) Agglutination: irregular antibody, "present"

(b) No agglutination: irregular antibody, "none detected"

K. Student's Questions

III. SUMMARY (5 min)

A. Review of Main Points

1. Blood donor reception

2. Blood donor records

3. Blood donor equipment

4. Phlebotomy

5. Blood collection

6. Film

7. Blood storage, issue and transportation

8. Blood components

9. Precautions

10. Blood processing

B. Closing Statement: It will be your responsibility as a blood bank technician to observe all precautions, maintaining rigid laboratory controls and accurate records. Every technique specified must be employed to assure that the blood the patient receives is the best possible so that the patient's condition is not jeopardized by his receiving blood improperly procured, processed or stored.

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ANNEX A
INSTRUCTIONAL AIDS

VIDEOTAPE

VT 12, 29:00 min.	BLOOD DONATION. A demonstration of the techniques of handling a volunteer blood donor and the collection of a unit of whole blood. This includes use of equipment, performing a donor physical, preparation of the puncture site, phlebotomy, collection of the unit of blood and pilot specimens, proper identification, precautions against donor reactions, and interim storage and handling of the unit of blood.

NOTE: This videotape is shown in part II.F.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *IM 08-280-108
LABORATORY SCIENCES DIVISION 046
CLINICAL MICROSCOPY BRANCH

Blood Donor Processing

Human blood for transfusion is the most precious and at the same time, perhaps the most dangerous of any biological commodity routinely used in the practice of medicine today -- precious because it can be manufactured only by the human animal, and dangerous because once transfused into a patient it cannot be retrieved. It may be part of your duties as a laboratory technician to recruit blood donors, perform the phlebotomy to obtain the unit of blood, and perform the necessary processing to insure that the unit obtained is suitable for transfusion. Each step, from the initial donor interview through the processing to the final placement of that unit in the blood bank must be done correctly and with absolute accuracy. Truly, the operation of a blood bank must be a "zero defects" program!

When the prospective donor enters the blood bank for the first time he may be apprehensive to the point where there will not be a second time if you do not perform your job in the best possible manner. Initial impressions for the "first time" donor are usually long lasting; therefore, you must conduct yourself in a most professional manner, while remaining friendly and making the prospective donor comfortable so that he will wish to return for future donations.

Maintaining scrupulously accurate records and files on both donors and recipients are of paramount importance in the blood bank. Accurate records insure positive identification of all specimens and units of blood, and should help to preclude the accidental transfusion of an inappropriate unit of blood to the recipient. Accurate blood bank records begin with DD Form 572 (Blood Donor Record Card) which is completed by each prospective donor prior to donation. In addition to being a record of the donation, this form may be used to establish and maintain a "walking blood bank", or file of prospective donors according to blood group and type for use in crisis situations.

The preliminary information blocks on the DD Form 572 identify the donor: name, grade, social security account number, sex, organization, address and telephone number.

The medical history portion of the DD 572 is in the form of a questionnaire. All questions must be answered with either a "yes" or "no" response. Explanations must be recorded for any "yes" response. This questionnaire, when properly and honestly completed by the donor, is designed to protect both the prospective donor and all possible recipients. An affirmative response to any question may be cause to reject a prospective donor, either temporarily or permanently.

A prospective donor with a history of malaria shall be deferred for three years after becoming asymptomatic and cessation of therapy. An immigrant or visitor from an area considered endemic for malaria may be accepted as a blood donor after three years after departure from the area. Prospective donors who have taken antimalarial prophylaxis or who have been military personnel in an area considered endemic shall be deferred for three years after cessation of therapy and/or departure from the area if they have been asymptomatic in the interim--travelers to and temporary residents of malaria endemic areas are deferred for six months, provided they are asymptomatic and have not taken antimalarial therapy. Donors whose blood is to be used for the preparation of plasma, plasma components or fractions devoid of intact erythrocytes may be excluded from these restrictions.

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*This instructor's manuscript supersedes IM 08-280-108, 034.

Any individual with a history of viral hepatitis, jaundice, or a positive test for HAA is permanently rejected as a donor due to the danger of transmitting hepatitis. Persons who have had close physical contact with hepatitis patients are deferred for a period of six months. Similarly, a prospective donor with tattoos must be deferred for six months from the date of tattooing due to danger of exposure to hepatitis from nonsterile tattoo needle or ink. Drug addicts are rejected due to the dangers from contaminated needles, especially hepatitis.

Inoculations and vaccination will disqualify prospective donors for various lengths of time (dependent upon what immunization was received) due to anamnestic antibody response mechanisms. (Note: for precise deferment time in specific instances consult current, authoritive references, e.g., TM 8-227-3 or AABB Technical Methods and Procedures.) In the case of blood group antigens, prospective donors may be acceptable provided an appropriate notation is made, and at least 6 months have elapsed since immunization last occurred.

The conditions explicitly described serve only as examples of reasons for rejecting the prospective donor and are by no means complete. Any doubts of eligibility should be referred to the laboratory NCOIC, laboratory officer or medical officer.

The physical examination consists of a number of evaluations which may give important information concerning the prospective donor's state of health. The individual's general appearance is evaluated and conditions such as pallor are noted. The prospective donor must be rejected or deferred if he fails to have favorable responses in all blocks in the physical examination portion of the DD Form 572.

All prospective donors must weight at least 110 pounds for the donation of a full unit (450 ml) of blood. Donors weighing less than 110 pounds may be bled proportionately less, provided that the volume of blood collected is at least 90 percent of the volume required for the anticoagulant solution (i.e., not less than 405 ml). This amount can be calculated by using the following formula:

$$\frac{\text{weight of donor X 450}}{110} = \text{ml of blood that may be drawn}$$

If this calculated volume of blood is less than 405 ml, the prospective donor must be rejected.

The prospective donor's oral temperature shall not exceed 99.6° F (37.5° C).

The pulse must be between 50 and 100 beats per minute with a regular rhythm. Abnormalities must be evaluated by the medical officer.

The systolic blood pressure shall be between 90 and 180 mm of mercury, with the diastolic pressure should not exceed 100 mm. Abnormalities must be evaluated by the medical officer.

Inspect both arms of the prospective donor for needle marks suggestive of drug abuse. If there is any suspicion of narcotic use the medical officer must be consulted.

The prospective donor must be evaluated by means of either hematocrit or hemoglobin levels. Male donors must have a minimum hemoglobin of 13.5 gm/100 ml; female donors may be accepted with a minimum hemoglobin of 12.5 gm/100 ml. This minimum hemoglobin concentration may be determined by the specific gravity (copper sulfate) method. The copper sulfate solutions have specific gravities of 1.055 (male) and 1.053 (female). Drops of blood containing the minimum or above minimum amount of hemoglobin will sink in copper sulfate within 15 seconds when dropped from an appropriate height. Male donors may have a minimum hematocrit of 41 percent, and females 38 percent when determined in the usual manner.

The prospective donor must be 17 thru 65 years for individuals on active military duty. For legal reasons, donations by civilian minors 18 thru 65 are accepted in accordance with prevailing standards.

No individual may donate blood unless at least eight weeks have elapsed since the last donation. No more than five donations may be permitted by a donor within any twelve month period unless permission of a physician is obtained.

After all of the questions have been answered by the donor and the physical examination is completed, insure that the prospective donor has signed the release portion of the DD 572. This is a legal release by the donor stating all questions in the medical history questionnaire have been answered correctly. The donor affirms that the donation is voluntary and allows the blood to be used as needed, without restrictions.

The plastic bag has replaced glass bottles as the standard container for the collection of blood. The most commonly employed anticoagulant is acid, citrate, and dextrose (ACD). The acid reduces the pH; citrate prevents coagulation by binding ionic calcium in the plasma; and the dextrose nourishes the cells while retarding cellular deterioration.

The plastic bag offers many advantages over the glass bottle for the collection of donor blood. The bag is a "closed system," with its integral collection tubing and phlebotomy needle; thus its use reduces the chance of contamination. The plastic of the bag is smoother and less porous than glass of the bottle, so that platelets are better preserved. The bags are normally filled by gravity instead of vacuum, so cellular elements are less likely to be ruptured traumatically. Glass bottles break easily, while plastic bags are resilient. Rigid bottles take up more storage space than flexible plastic bags.

Disadvantages of the plastic bag are few but significant. The bags may be overfilled when the donor's blood is collected. The tubing on the plastic bags must be sealed carefully so that the contents are sealed in and not contaminated.

The glass bottle may be gravity flow (which fill by gravity) or vacuum type (which fill more rapidly since there is negative pressure in the bottle). Any anticoagulant may be used in the glass bottle; however, acid citrate dextrose is still the most common anticoagulant routinely employed. Advantages of the glass bottle include the fact that they are difficult to overfill; the pilot tubes are affixed more readily; and there is no integral tubing to be tied off or sealed. Disadvantages include bulkiness, fragility tendency to lyse more cells and platelets than plastic bags, and difficulty in removing blood components from the bottle.

Once the donor has met all of the donor requirements, he is ready to be prepared for the phlebotomy and donation of blood. Assist the donor to the phlebotomy table or bed. The donor should remain in a comfortable supine position throughout the donation and for a few minutes thereafter.

To select the most suitable vein for donation, palpate the veins in the antecubital fossa of both arms. The vein selected should be large enough to permit proper entry of the needle, allowing the blood to flow rather rapidly without collapsing the vein. Palpation should not only locate the most suitable vein, it must also determine the size, depth, and direction or course of the vein.

A five-step preparation of the skin surrounding the proposed venipuncture site must be accomplished to assure that the phlebotomy is performed aseptically. After the proposed site of phlebotomy has been selected, the tourniquet is released. Apply surgical soap (approximately 15% aqueous solution of USP green soap or the equivalent) scrubbing vigorously over an area at least 2 1/2 inches in each direction from the puncture site, using forceps stored in antiseptic for a period of at least thirty seconds with gauze (or sixty seconds with cotton balls) to remove dirt, fat, oils, desquamated cells, etc.

Apply ten percent acetone in seventy percent alcohol (nine parts seventy percent ethyl or isopropyl and one part acetone) with forceps, using gauze or cotton, to prepare the skin for the iodine solution which follows. Apply tincture of iodine (three percent with seventy percent alcohol) with sterile, cotton tipped applicator sticks. Begin at the site of intended phlebotomy, and apply the iodine in a concentric spiral beginning at the center to cover an area at least 1 1/2 inch radius in all directions. The iodine must be allowed to remain until it has dried to allow time for the solution to penetrate to its fullest depth (Note: Some persons are allergic to iodine; therefore, all donors should be questioned prior to its application.) Apply ten percent acetone in seventy percent alcohol to remove iodine solution which has now served its purpose; again, use the concentric spiral pattern to prevent contamination. Finally, cover the phlebotomy site with sterile, dry gauze to prevent contamination. Never allow anything that is not sterile to touch the phlebotomy site after it has been prepared.

Apply a tourniquet or sphygmomanometer cuff to the upper arm tightly enough to occlude venous return but not so tight as to stop arterial flow; this will distend the veins and make them stand out. A loose overhand knot should be tied closely to the needle to allow for as many tubing segments as possible. Remove the cover from the sterile needle and the sterile covering from the phlebotomy site, and promptly insert the needle into the vein, so that it is well within the lumen of the vein. The needle should be taped to the arm and covered with sterile gauze.

During the donation period a pleasant, friendly atmosphere should be maintained. The donor must never be left unattended. The bag must be tilted and flexed throughout the donation to insure thorough mixing of the blood and anticoagulant; mixing is recommended at least twice per minute. Even when donor scales are used, the entire operation must be closely watched to prevent overfilling of the bag.

Once the unit is drawn, the loose overhand knot in the integral tubing is drawn into a "white" knot, a set of hemostatic forceps applied between the knot and the needle, and the tubing is cut between the knot and forceps. The pilot tubes (two to four, depending on the laboratory SOP) are filled from the tubing by releasing the forceps while the needle is still in place. It is critical that all identification be verified at this time by checking all identifying information on the DD form 572, the blood bag and the pilot tubes to insure that they agree.

The tourniquet or sphygmomanometer cuff is removed, the tape removed, and the needle withdrawn. Pressure must be applied immediately after the needle is withdrawn; the donor must hold up his arm and continue the application of pressure on the venipuncture site. (Note: do not allow the donor to bend his arm.) More often than not, bending the arm instead of applying finger pressure over the site will result in a hematoma. The patient should remain in the supine position for ten minutes after the donation is completed. After ten minutes, help him sit with his legs over the edge of the bed; if he feels no ill effects, help him off the bed. He should be assisted into another room (out of sight from the bleeding area) and given some refreshments. After the donor has relaxed and been observed for any reactions or symptoms, he may be dismissed.

Before, during and after donating, a reaction may occur. Some of the more common reactions are: (1) syncope (fainting), (2) nausea and vomiting, (3) convulsions, and (4) hematoma. If any reaction should occur during the donation, discontinue the donation immediately and treat accordingly. Any donor reaction should be followed up and a decision made as to whether or not he should be excluded from future donations.

Once the blood has been collected it must be refrigerated immediately. Blood storage refrigerators should contain nothing but blood, blood components, reagents and samples for blood bank work. Separate areas of blood storage should be designated and labeled for unprocessed blood, available (processed and labeled) blood, cross-matched blood and quarantined blood. If donor blood (anticoagulated with ACD) is properly collected and refrigerated, it may be stored for up to 21 days, at which time at least seventy percent of the erythrocytes should remain viable. The refrigerator should operate within a two degree range between 1 and 6° C (33.8 and 42.8° F), under continuous monitoring in such a manner that temperature fluctuations are recorded on a chart. The refrigerator must have an alarm system capable of alerting personnel when the temperature reaches a low of 1 degree Centigrade or a high of 6 degrees Centigrade.

All units of blood in the blood bank should be inspected daily for the presence of hemolysis, clots and/or contamination. During this inspection, units are rotated by date (so that the units closest to their expiration date will generally be used first) with expired and/or unsuitable units removed for proper disposal.

The issuance of blood from the Blood Bank must be accomplished under the most rigid laboratory controls. This is the final opportunity to audit the procedures and identification before the blood is transfused to the recipients. Accurate records of donor, and recipients, compatibility test results, issuances and requests must be maintained. It is crucial that all records and means of identification be verified by checking each one in turn and comparing each step with the last; remember that the vast majority of transfusion reactions result from administrative rather than technical errors. Check the donor name and number with that on the unit of blood, pilot tubes, SF 518 (Blood Transfusion) and the Blood Bank records against the recipient's full name and identifying number. The unit of blood must be inspected to insure that there is no hemolysis, clots or contamination. Finally, insure that all steps of the cross-match have been performed properly and all forms are executed completely and accurately.

When it is necessary to ship blood to another installation, the Blood Bank personnel must insure that DD Form 573 (Shipping Inventory of Blood Collections) is completed in detail. Items of particular importance are:

(1) the organization that is shipping the blood, (2) the organization that will receive the blood, (3) the hour and the date that the shipment is made, (4) the name and type of carrier transporting the blood, (5) the train or flight number (if known), (6) accurate inventory by serial number of all units of blood included in the shipment, and (7) notation under the remarks section of the DD form 573 noting the time the shipment was prepared.

Blood shipments should be made only in containers specifically designed and labeled for shipment of human blood. Blood shipment containers are sturdy and well insulated for protection of the blood; and clearly labeled as to contents and what special handling and re-icing instructions are needed.

The temperature of the blood must be kept between 1 and 10 degrees Centigrade (33.8 and 49.9° F). Refrigeration during shipment is usually accomplished with the "wet ice." While the usual consideration is to keep blood from becoming too warm, it may also be necessary to keep it from getting too cold on some occasions. Dry ice or super-cooled wet ice must NOT be used in shipping or storage of whole blood or cells because of the danger of freezing the cells, with subsequent hemolysis. The shipment container is clearly labeled as to requirements for re-icing and inspection of contents in the event that shipment is not completed within 24 hours.

Frequently the physician may order blood components when treating patients. In addition to whole blood, these components include fresh frozen plasma, platelet rich plasma, platelet concentrations, leukocyte-poor blood and packed red cells. The use of components allows the physician to selectively administer only the blood fractions necessary for treatment of the patient's condition.

Precautions must be initiated, observed and complied with in handling human blood. Select only competent personnel who are fully trained and responsible for employment in all phases of procurement, processing, storage and issue of human blood. Accurate records of all phases of blood bank operations must be maintained, both from the medical-legal aspect and in the interest of the patient and laboratory. Rigid laboratory controls will reduce chances of error, and insure that all test results are valid. Pyrogen-free solutions and standardized biological reagents should be used to insure test validity. Blood should never be frozen; it is warmed only in exceptional cases. Each time blood is issued, a checklist for issuing blood should be consulted and followed to insure that all checks and inspections have been carried out and no discrepancies of any kind exist.

Processing of all units of blood must be accomplished and the units properly and accurately labeled before they are ready to be used for transfusion. Routine processing should include ABO grouping, serum grouping, Rh typing, a serological test for syphilis, a test to detect the presence of hemolytic Anti-A and/or Anti-B (on units except those of group AB), a test for detection of hepatitis-associated (Australia) antigens, and an antibody screening test for the detection of irregular antibodies. The results of all testing will be included on the label of the unit of blood, on the DD Form 572, and all other appropriate records being maintained. Again the numbers must be checked and the unit and records verified to insure all necessary information is present, correct, and properly entered.

A modified indirect Coomb's test (antibody screen) is performed to detect atypical antibodies. Commercially available group "O" screening cells, containing the broadest cross-section of antigens possible, should be used for the detection of the maximum number of antibodies. Additionally, anti-human globulin sera will be required for completion of testing. As with all Blood Bank testing, both positive and negative controls should be run to insure the validity of test results.

The three phase procedure for the antibody screening is outlined as follows:

(1) Label one tube (12 x 75 mm) with donor's identification.

(2) Place one to two drops of the donor's serum in the tube.

(3) Add one to two drops of screening cells.

(4) Serofuge for fifteen seconds and read for agglutination.

Most saline reactive antibodies should be detected at this point.

(5) The cell button is then completely resuspended by vigorously shaking the tube.

(6) The test is converted to the protein phase by the addition of two drops of 22 percent bovine albumin.

(7) Serofuge for fifteen seconds and read the unincubated protein phase for agglutination.

(8) Resuspend the cell button completely prior to incubation.

(9) Incubation at 37° C for fifteen to sixty minutes is required to allow time for attachment of any antibodies present to the antigen sites.

(10) After the incubation period the specimen is again Serofuged fifteen seconds and read for agglutination to determine if any protein reactive antibodies are present.

(11) The specimen is then washed three to five times with saline to remove any excess proteins.

(12) The specimen is converted to the Coomb's phase at this point by

adding two drops antihuman globulin.

(13) The specimen is Serofuged fifteen seconds and read for agglutination.

If the agglutination is detected in any phase of the antibody screening, the notation "present" is made since irregular antibodies have been detected by the reaction agglutination. No agglutination indicates a negative test result; however, it does not necessarily indicate that irregular antibodies are absent, but rather that they were not detectable by our methodology. Therefore the test results are reported as "none detected."

It will be your responsibility as a Blood Bank technician to observe all precautions, maintaining rigid laboratory controls and accurate records. Every technique specified must be employed to assure that the blood the patient receives is the best possible, so that, the patient's condition is not jeopardized by his receiving improperly processed or stored blood.

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Outline of Procedures Prior to Blood Transfusion

The purpose of this summary is to outline necessary transfusion service procedures prior to issue of blood for transfusion. These procedures must be followed exactly since any error carries with it the very real danger that death may result.

NOTE: The manufacturers' instructions included with reagents should always be followed exactly, even if they disagree somewhat with the general instructions summarized below.

I. ABO Blood Grouping Procedures

A. Cell Grouping

Cell grouping can be performed using either slide or tube technique. The slide technique, to be reviewed first, is considered less sensitive than the test tube method.

1. Slide Blood Grouping Method

a. Apparatus

(1) Glass microscope slides

(2) Wooden applicator sticks

b. Reagents

(1) Anti-A grouping serum

(2) Anti-B grouping serum

(3) Anti-A, B grouping serum

NOTE: These sera must meet the sensitivity, avidity, and specificity standards of the National Institutes of Health.

c. Technique

(1) Using a wax pencil, divide a glass microscope slide in half by drawing a line through the center across the short axis.

(2) Mark the left half of the slide "A" and the right half "B". Identify the slide with the patient's name or blood bank code number.

(3) Place 1 drop of anti-A serum in the square marked "A" and 1 drop of anti-B in the square marked "B".

(4) With the aid of a wooden applicator stick and using whole blood (capillary puncture), clotted blood, or whole blood (venipuncture) with anticoagulant, transfer a small amount (no greater than half the antisera volume) of the blood specimen to the drop of anti-A serum.

(5) Mix in a circular pattern so as to spread the cells and serum in a circle approximately 25 mm in diameter.

(6) Using a clean applicator stick, transfer a small amount of the blood specimen to the drop of anti-B and mix as before. Be sure to use a clean applicator stick for each side of the slide.

(7) Tilt the slide so that the cell-antiserum mixture gathers in a drop at the lowest part of the circle and rotate gently.

(8) Agglutination usually occurs within a few seconds. In all cases, readings must be made within 3 minutes.

NOTE: Anti-A and anti-B react best at 4°C. Do not apply heat to the slide in ABO testing. The reaction will usually be rapid and strong at room temperature, but all negative readings with anti-A typing serum must be confirmed with anti-A, B serum used in the same manner as described above.

2. Test Tube Blood Grouping Procedure

a. Apparatus

(1) Test tubes

(2) Disposable pipettes

(3) Serofuge

b. Reagents

(1) Anti-A grouping serum

(2) Anti-B grouping serum

(3) Anti-A,B grouping serum

(4) Normal saline (0.85 percent)

c. Technique

(1) Select three scrupulously clean test tubes; label "A", "B", and "A,B". Write the patient's name or blood bank code number on each tube.

(2) Place 1 drop of anti-A grouping serum in tube A, one drop of anti-B grouping serum in tube B, and one drop of anti-A,B grouping serum in tube A,B.

(3) Add 1 drop of saline-suspended patient's cells (5 percent) to each tube.

(4) Mix tubes well and spin 15 seconds in the Serofuge.

(5) Gently dislodge the button of cells from the bottom of the test tube and check macroscopically for agglutination.

B. Serum Grouping (Confirmation Grouping)

From the basic reaction of the ABO blood groups, it is known that when an antigen is present on the red cell, the corresponding antibody is absent from the plasma or serum. Thus, tests using the individual's serum and known cells either confirm results or point out discrepancies in the tests based on the reaction of the person's cells with known antiserum.

1. Apparatus

a. Test tubes

b. Disposable pipettes

c. Serofuge

2. Reagents

   a. Known group A cells

   b. Known group B cells

NOTE: Above cells are prepared in a 5 percent suspension in normal (0.85 percent) saline.

3. Technique

   a. Select two absolutely clean test tubes; label one "A" and the other "B". Write the patient's name or blood bank code number on each tube.

   b. Place 2 drops of the serum to be tested into each tube.

   c. Add 2 drops of a freshly prepared 2 to 5 percent saline suspension of known pooled group A cells to tube marked "A".

   d. Add 2 drops of a freshly prepared 2 to 5 percent saline suspension of known pooled group B cells to tube marked "B".

   e. Mix the tubes well.

   f. Centrifuge tubes for 15 seconds in a Serofuge.

   g. Check macroscopically for agglutination.

Cell grouping

Blood Cells from Group:	Reaction to Anti-A serum	Reaction to Anti-B serum	Reaction to Anti-A,B Serum
0	No agglutination	No agglutination	No agglutination
A	Agglutination	No agglutination	Agglutination
B	No agglutination	Agglutination	Agglutination
AB	Agglutination	Agglutination	Agglutination

Serum grouping

Blood Group	Antibody in Serum	Reaction with Group A Cells	Reaction with Group B Cells
A	Anti-B	No agglutination	Agglutination
B	Anti-A	Agglutination	No agglutination
AB	None	No agglutination	No agglutination
O	Anti-A & Anti-B	Agglutination	Agglutination

II. Rh Typing Procedures

The Rh system differs from the ABO system in that it has no naturally occurring antibodies. The term "Rh" refers in its broadest sense to six different genetic factors and many variants which are all antigenic.

As with all immunohematological procedures, a double-check system is used in performing Rh typing procedures. Use two different lot numbers of typing serum and perform test in duplicate if only the test tube method is employed. If only one lot number is available, both test tube and slide method is performed. The negative control, which consists of the patient's cells and bovine albumin, is run with each test. This is necessary, since some patients may show false positive results because of the bovine albumin in the Rh test serum suspension.

A. Slide Method for Rh Type

1. Apparatus
   - a. Microscope glass slides
   - b. Medicine droppers or straws
   - c. Applicator sticks
   - d. Rh viewbox
2. Reagents
   - a. Anti-Rh_o (anti-D) typing serum
   - b. Normal saline (0.85 percent)

3. Technique

   a. Carefully label a clean glass slide with the patient's name or blood bank code number.

   b. Place 1 drop of typing serum on the slide.

   c. Place 2 drops of the whole blood to be tested beside the drop of typing serum.

   d. Mix the cells and antiserum with an applicator stick.

   e. Place the slide on a warm viewbox (45 to 50°C).

   f. At 10-second intervals, tilt the viewbox and the slide so that most of the liquid accumulates at one end of the slide, then return the slide to the horizontal position.

   g. Examine for macroscopic agglutination within 2 minutes. Agglutination of the specimen indicates the presence of the Rh_o antigen.

B. Test Tube Method for Rh Typing

1. Apparatus

   a. Test tubes

   b. Applicator stick

   c. Serofuge

2. Reagents

   a. Anti-Rh_o (anti-D) typing serum

   b. Normal saline (0.85 percent)

   c. Bovine albumin (22 percent)

3. Technique

   a. Label one test tube with the patient's name or blood bank code number and the letter "D". Place in the tube 1 drop of anti-Rh_o (anti-D) typing serum (test).

b. Label a second test tube with the patient's name or blood bank code number and the letter "C". Place in the tube 1 drop of bovine albumin (control).

c. Dip an applicator stick into blood cells from a clot of whole blood and transfer the red cells adhering to the applicator stick to the typing serum in the test tube. Repeat this procedure, using a clean applicator stick, for the control tube. This procedure results in approximately a 2 to 5 percent suspension of red blood cells in each tube.

d. Centrifuge for 15 seconds in the Serofuge.

e. Loosen the packed cell button in the bottom of each tube by gentle agitation and observe macroscopically for agglutination.

f. If the tube marked "C" shows agglutination the test must be repeated using "Saline anti-Rh_0" and the procedure outlined with this reagent.

g. Rh positive specimens show agglutination in the tube marked "D" and no agglutination in the tube marked "C".

C. Du Factor Technique

1. If no agglutination occurs in the test tube method for Rh typing, incubate the tube for 15 minutes in a 37°C water bath or heating block.

2. After incubation, remove the tubes from the incubator and wash the cells three to four times with normal (0.85 percent) saline. Use fresh saline for each wash. Decant the supernatant after the last washing, removing as much of the saline as possible.

3. Add 1 drop of antihuman globulin (Coombs) serum.

4. Centrifuge the tube for 15 seconds in the Serofuge.

5. Resuspend the packed cells by gentle agitation and examine macroscopically for agglutination.

6. No agglutination in tube "C" with agglutination in tube "D" indicates the blood is Rh positive.

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7. Add 1 drop Check Cell (known Coombs positive) to all tubes showing no agglutination. Centrifuge 15 seconds in Serofuge. Agglutination must be present or test is invalid.

NOTE: Rh typing procedures for other antigens in this system are performed with appropriate typing serum and procedure as outlined for Rh test tube method of typing.

III. Antihuman Globulin (Coombs) Test

A. The Direct Antiglobulin (Coombs) Test

This test is employed when it is suspected that the patient's red blood cells have been coated with an antibody in the patient's own body (in vivo). Clinical suspicion of erythroblastosis, hemolytic anemia, or a hemolytic transfusion reaction warrants performance of this test. As a screening procedure, the direct antiglobulin test is excellent; however, a negative test does not rule out the possibility that such a reaction did in fact occur.

1. Apparatus
   a. Test tubes
   b. Applicator sticks
2. Reagents
   a. Antihuman globulin (Coombs) serum
   b. Normal saline (0.85 percent)
   c. Check Cells (Coombs positive control)
3. Technique
   a. Dip an applicator stick into blood cells from a clot of whole blood and transfer the red cells adhering to the applicator stick to a drop of saline in a test tube.

NOTE: A negative control should accompany each test, and a positive control each set of tests.

   b. Fill the tube approximately three-fourths full with fresh saline, mix, centrifuge for 1 minute and decant. Repeat the washing at least four times. Decant supernatant completely after final wash.

c. Add 1 drop of antihuman globulin (Coombs) serum.

d. Centrifuge for 15 seconds in Serofuge.

e. Gently mix contents of tube and observe for agglutination which occurs when *in vivo* sensitization has occurred.

f. Run Check Cells on all negative tests to insure addition and reactivity of Coombs Serum.

B. The Indirect Antihuman Globulin (Coombs) Test

This test is employed when a screening test is desired for the demonstration of unexpected antibodies in a patient's serum or for the demonstration of an antigen on a patient's red blood cells. This test is particularly useful in prenatal studies. It detects *in vitro* coating of the red blood cells.

1. Uses of the indirect Coombs test

   a. With known antisera--to test for antigens on a patient's red blood cells.

   b. With known test cells--to test for circulating antibodies in a patient's serum.

   c. With unknown cells and unknown serum--to test for corresponding antigen(s) and antibody(s) in a test system. This is the test system utilized in a crossmatch.

2. Apparatus

   a. Test tubes

   b. Applicator sticks

3. Reagents

   a. Antihuman globulin (Coombs) serum

   b. Normal saline (0.85 percent)

   c. Known antiserum or test cells as applicable

   d. Check Cells (Coombs positive control)

4. Technique

   a. Prepare a 5 percent suspension in fresh normal saline of the selected test cells.

b. Place 2 drops of serum in a properly labeled test tube.

c. Add 2 drops of the prepared cell suspension to the tube.

d. Incubate in a 37°C water bath or heating block for 30 minutes.

e. Remove the tube from the incubator, wash four times, add 1 drop of Coombs serum, and centrifuge 15 seconds in Serofuge.

f. Agglutination indicates that an antibody or antibodies have reacted with the specific antigen or antigens on the test red blood cells.

g. Run Check Cells on all negative tests to insure addition and reactivity of the Coombs serum.

IV. Screening Procedures for Unexpected Antibodies

This test procedure shall be a part of all donor and recipient blood processing prior to the administration of blood or blood products to a patient, as a screen for unexpected circulating antibodies. The test cells are usually commercially prepared and will contain all clinically significant antigens which may cause transfusion reactions.

A. Apparatus

1. Test tubes
2. Applicator sticks
3. Disposable pipettes or straws

B. Reagents

1. Bovine albumin
2. Normal saline (0.85 percent)
3. Antihuman globulin (Coombs) serum
4. Reagent red blood cells (screening cells)
5. Check Cells (Coombs positive control)

C. Technique

1. Saline phase:

a. Place 1 drop of patient's serum in a properly labeled test tube. (I,II,IA,IIA)

b. Add 1 drop of a 3 to 5 percent suspension of reagent red blood cells.

c. Centrifuge tubes I and II for 15 seconds in Serofuge and observe for agglutination.

d. Incubate them for 30 minutes at room temperature.

e. Centrifuge for 15 seconds in Serofuge and observe for agglutination.

2. Albumin phase:

a. Add 2 drops of bovine albumin, mix gently, centrifuge for 15 seconds, and observe for agglutination.

b. Incubate for 20 minutes at 37°C in an incubator.

c. Remove from incubator, centrifuge for 15 seconds, and observe for agglutination.

3. Coombs Phase:

a. Wash four times, add 1 drop of Coombs serum, and centrifuge 15 seconds in Serofuge.

b. Agglutination in any phase indicates that an antibody has reacted with its corresponding antigen on the reagent red blood cells. If this test serum is from a donor, the unit of blood is unsuitable for administration. If the test serum is from a recipient, a panel of cells is utilized for identification of the antibody, and all donor units must be screened for the corresponding antigen prior to their administration. This screening procedure is outlined as a possible use of the indirect Coombs test (see page 9).

V. Antibody Identification Procedure Utilizing Panel of Reagent Cells.

This procedure is identical to the screening procedure outlined above with the exception that a number of known reagent cells (8 to 16) are utilized. By noting the tube number(s) in which a reaction occurred, it is possible to identify the antibody in the patient's serum.

A chart is furnished by the manufacturer showing antigens on each cell in the panel, and the reactions which would be expected to occur by cell number with any antibody of medical importance.

In addition to the antibodies of the ABO blood groups and the Rh blood types, some of the important antibodies of the other blood groups include those of the Kell, Duffy, Kidd, and Lewis blood group systems.

VI. The Crossmatch Procedure

In the preceding pages of this summary, procedures for ABO grouping, Rh typing, direct and indirect Coombs test, and antibody screening and identification procedures have been discussed. These procedures are not an end in and of themselves, but rather a means to an end, which is to make the transfusion of blood as safe as possible.

The major cause of transfusion reactions which result in the death of the patient, however, is human carelessness, rather than errors in any of the foregoing procedures. The patient receives a unit of blood that was intended for someone else.

Prior to crossmatching, it is necessary to obtain samples of blood from the intended recipient, the patient. Forms requesting blood samples from the recipient must include his full name, hospital number, ward or room number, sex, social security number, and amount of blood desired. Forms that are incomplete in any respect will not be accepted by the blood bank staff.

Blood samples should be obtained in stoppered tubes and identified with a firmly attached label bearing the recipient's full name, hospital number, and the date of the phlebotomy. This label must be attached to the tube before leaving the bedside.

A. Apparatus

1. Test tubes

2. Disposable pipettes or straws

3. Applicator sticks

B. Reagents

1. Bovine albumin

2. Normal saline (0.85 percent)

3. Antihuman globulin (Coombs) serum

4. Check Cells (Coombs positive control)

C. The crossmatch consists of two parts, the major and minor sides.

The following procedure is a 4-tube, three-phase crossmatch.

1. Saline phase

a. Major Side: Recipient's serum--1 drop
Donor's cells--1 drop of 3 to 5 percent suspension of cells in saline.

b. Minor Side: Donor's serum--1 drop
Recipient's cells--1 drop of 3 to 5 percent suspension of cells in saline.

c. Mix gently and centrifuge 15 seconds in Serofuge.

d. Examine macroscopically for hemolysis or agglutination.

e. Incubate 30 minutes at room temperature.

f. Centrifuge 15 seconds in Serofuge.

g. Examine for hemolysis or agglutination.

NOTE: Hemolysis or agglutination in any phase of the crossmatch indicates an incompatibility. The unit of blood is not suitable for administration to that recipient.

2. Protein Phase

a. Set up major and minor sides as noted above.

b. Add 2 drops of bovine albumin to each tube.

c. Mix gently and centrifuge 15 seconds in Serofuge.

d. Examine macroscopically for hemolysis or agglutination.

e. Incubate at 37°C for at least 20 minutes.

f. Remove from incubation, mix gently, and centrifuge 15 seconds in Serofuge.

g. Examine macroscopically for hemolysis or agglutination.

3. Coombs Phase

   a. Use the same tubes from the Protein Phase and continue with them.

   b. Wash cells 4 times, add 1 drop of Coombs serum, and centrifuge 15 seconds in the Serofuge.

   c. Examine macroscopically for hemolysis or agglutination.

   d. Run Check Cells on all negative tests to insure reactivity of the Coombs Serum.

D. Discussion

If no evidence of hemolysis or agglutination is found in any phase, the crossmatch is certified as compatible, and that unit of blood is considered satisfactory for administration to that recipient. If the recipient will be given blood on a number of successive days, a new crossmatch must be performed each day a transfusion is to be administered since blood given on a preceding day may elicit an antibody response even though it was compatible in the crossmatch. The danger of a hemolytic transfusion reaction is thus minimized, though by no means completely removed. Remember that human error, especially clerical errors, are by far the greatest source of transfusion problems. Extreme care must be exercised at all times to prevent the accidental administration of blood or blood products to any person other than the one individual for whom it was intended. To insure that all procedures were performed correctly before the product is released to a prospective recipient, extreme diligence must be exercised in the completion of the practical work and completion of the paper work.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
CLINICAL MICROSCOPY BRANCH

*LP 08-280-101
068

Immunohematological Techniques (40 hours)

COURSES PRESENTED TO: 311-29B20 Medical Laboratory Specialist (Basic)

PLACE: Classroom

REFERENCES:

TM 8-227-3

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: None

PERSONNEL: One primary instructor (MOS 92B4H, 92B3H); two secondary instructors (MOS 92B4H, 92B3H)

INSTRUCTIONAL AIDS: None

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Practical exercise

I. INTRODUCTION: (5 min)

A. Opening Statement: Your ability to perform test procedures in the blood bank will depend on your ability to work with your hands in addition to understanding the principles behind the various test procedures. Any time you as a laboratory technician are called upon to perform any task in the blood bank, it will be your responsibility to insure that all procedures are performed accurately, that the results of testing are recorded properly, and that all precautions are strictly adhered to. It is imperative that you learn to perform all procedures with the utmost accuracy, since they are the basis of the hospital's blood bank.

B. Objectives

1. Perform tests of an immunohematological nature (ABO grouping, Rh_o (D) determination, and compatibility test procedures) under laboratory conditions, using the proper technique.

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*This lesson plan supersedes LP 08-280-101, 075.

2. Interpret results and report the various procedures in correct format during a proficiency survey.

C. Class Procedure and Lesson Tie-in

1. Forty hours of practical exercise in immunohematology.

2. Conferences as necessary to solve individual problems.

II. APPLICATION: (1785 min)

A. Arrangement of Students

Students will work at their regularly assigned are during the practical exercise.

B. Instructions to Students

1. Pick up blood specimens from instructor.

2. Pick up reagents and equipment for exercise from instructor.

3. Check chalkboard in classroom at the beginning of each period for special instructions.

4. Request assistance from instructor when needed.

5. Report results of testing on report form(s) provided by instructor.

C. Instructor's Activities

1. Prior to class

a. Prepare reagents as needed

b. Prepare and key unknown specimens for student analysis

2. During class

a. Issue materials, reagents and specimens as necessary

b. Monitor student's performance of procedures closely, making immediate corrections and answering student questions.

c. Check completed report forms and discuss problem areas with students

d. Maintain classroom control

D. Student's Activities

1. Under instructor's supervision, students will perform:

a. ABO grouping

b. RH_o (D) determination

c. Compatibility test

2. Student will be responsible for maintenance of individual equipment.

3. Student will record the results of daily practical exercise on form(s) provided, and hand them in to be checked by instructor.

E. Questions From Students

III. SUMMARY: (10 min)

A. Review of Main Points

1. ABO grouping

2. RH_o (D) determination

3. Compatibility test

B. Closing Statement: How many mistakes did you make in this practical exercise? If you consider each mistake as a seriously injured or lost patient you immediately understand why these procedures must be performed without error in the clinical laboratory.

INSTRUCTIONAL HOURS

Number of instructors required - - - 3

Primary hours	40
Secondary hours	80
Total	120

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
CLINICAL MICROSCOPY BRANCH

M 08-280-101
015

A Manual in Basic Immunohematological Procedures

INTRODUCTION:

Blood for transfusion is a biologically active therapeutic substance. It has specific effects on the human organism and dosage requirements just as any other therapeutic substance. It differs, however, from other "biologicals" in that it must be obtained from healthy individuals of the human race. At our present state of knowledge, whole human blood is a perishable substance, and being derived from human beings, must be collected in thousands of widely spread laboratories rather than being produced in a handful of quality, well-regulated laboratories, as are other biologicals. THE RESPONSIBILITIES OF THOSE WHO COLLECT, PROCESS, AND ISSUE WHOLE BLOOD ARE TREMENDOUS.

MENDELIAN THEORY:

According to genetics, the basic element determining heredity is the gene, each gene having an individual function, different from that of all other genes. Hundreds of genes are strung like beads on chromosomes, of which human beings have 23 pairs, or 46 individual chromosomes in all; each pair is alike in size and shape. Each gene has a partner, or mate, at the corresponding locus on the other chromosome of a pair. Thus, all human cells (except germ cells) contain 23 pairs of chromosomes. Each pair is strung with genes; each gene has a mate, and the mates are found at specific designated loci on the paired chromosomes.

The growth of the body is governed by the division of individual cells, one cell becoming two, two becoming four, etc. Each time the cell divides, it produces a complete, equal "daughter" cell having the same number of genes, chromosomes, and other cellular elements as the "parent" cell. Thus, the number of chromosomes and genes in each cell remains constant. However, one of Mendel's laws states that in the life of the germ cell, one reduction division occurs; that is to say, the pairs of chromosomes separate so that the mature ovum and sperm each contains only 23 chromosomes, rather than 46, and only one of each of the paired genes. Thus, when the sperm and ovum unite, a new organism is formed with the normal 23 pairs of chromosomes, half from each parent. Chance alone determines which one of a pair of chromosomes may end up in the germ cell in a reduction division.

Variations among individuals are explained not only by the law of independent assortment, but also by the phenomena known as "crossover" and mutation. Crossing over is, more or less, one gene changing places with its partner on the other chromosome. Each of the paired genes has the same locus on

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*This mimeo supersedes M 08-280-120, 041, and M 08-280-106-2, 041.

its corresponding chromosome. When crossover occurs, the genes merely trade places. Normally, two paired genes never end up on the same chromosome. Mutation, on the other hand, involves a qualitative change in the gene itself. The change occurs suddenly and permanently. Actually, it amounts to the formation of a new gene, which may be passed down to future generations. The "new" gene occupies the same locus as the gene it replaces. Often, an individual may have a "new" gene on one chromosome and a normal "old" gene on the other.

Another important consideration in understanding genetics is allelomorphism. Alleles, or allelomorphs, are alternate genes which occupy the same locus. For example, there may be a variety of genes determining eye color, yet only one will appear on the specific locus on the chromosome. That locus will always be occupied by a gene for eye color, yet an alternate gene (or allele) may be introduced in future generations.

The terms phenotype and genotype should be carefully distinguished. Phenotype refers to demonstratable characteristics, while genotype refers to the sum total of the individuals genes. For example, a brown-eyed person would have the phenotype "Brown eyes positive," yet his genotype might include a gene for blue eyes which would be revealed only in future generations.

Another important characteristic of genes is dominance and recessiveness. A dominant gene will be revealed in the presence or absence of a recessive gene, but a recessive gene will reveal itself only in the absence of a dominant gene. For example, let "B" stand for the gene for brown eyes and "b" stand for the gene for blue eyes. Brown eyes are dominant to blue eyes so that the combination BB and Bb will both produce brown eyes. Blue eyes will be expressed only by the combination bb. A Bb father and a Bb mother, both brown-eyed, have a statistical chance of having one blue-eyed child to every three brown-eyed. The three brown-eyed children would have a phenotype of B, because of the demonstratable trait of brown eyes, but only one would have the genotype BB and the two others would be Bb. The blue-eyed child would have the phenotype b and the genotype bb.

Homozygous means the same genes and heterozygous means different genes. Thus, a BB person is homozygous for brown eyes, but a Bb person is heterozygous for brown eyes. A blue-eyed person, of course, must be homozygous because he must have the genotype bb in order to have blue eyes.

The subject of genetics fills many thousands of volumes and occupies the full time of many scientists, yet a knowledge of the foregoing basic principles is sufficient to lend a general understanding to the subject of blood groups and types.

AGGLUTINATION:

Agglutination is a phenomenon which occurs when blood cells clump together and settle out as a result of a reaction between specific antibodies in the serum and their specific antigens on the red cell surface. An antibody causing agglutination is referred to as an agglutinin. The antigen involved is called an agglutinogen. Agglutination is the reaction most commonly observed in blood banking procedures.

THE ABO BLOOD GROUP FACTORS:

Blood grouping is not an end in itself, but rather a means to an end. That end is to make the transfusion of blood as safe as possible. Before the discovery of the four major blood groups in 1900 and the Rh_o (D) factor in 1940, a transfusion of whole blood was surrounded with uncertainties and often resulted in death or permanent sensitization to the recipient. Today, the existence of four distinct blood factors in the ABO system is recognized. Two of these are antigens (agglutinogens) which are found on the red blood cells, and two are antibodies (agglutinins) which are found in the plasma or serum. There are only two of these factors present in any given blood at any time.

An ABO blood group is named for the antigen (agglutinogen) present on the red blood cell. Thus, group A blood has the A antigen on the red cell, group B the B antigen, group AB has both antigens, and group O blood lacks both antigens. In addition, ABO blood groups are normally further characterized or confirmed by the presence or absence of antibodies (agglutinins) in the plasma or serum. Group A blood has an anti-B antibody, group B has an anti-A agglutinin, group AB blood has neither antibody, and group O has both antibodies. Thus any given blood sample will yield two factors: an antigen and an antibody, two antigens, or two antibodies. But, they are named only for the antigens (or lack of them) present.

Table I

Antigen-Antibody Breakdown of the ABO Blood Groups

Blood Group Designation	Antigen(s) (Agglutinogen) Present on Red Cell	Antibody(s) in Serum (Agglutinin)
Group "A"	A antigen	B antibody
Group "B"	B antigen	A antibody
Group "AB"	A and B antigens	No antibodies
Group "O"	No antigens	A and B antibody

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LANDSTEINER'S RULE

The ABO system, and only the ABO system, may be summarized by the Rule of Landsteiner. It states: "IF AN ABO ANTIGEN IS PRESENT ON THE RED CELL, THE CORRESPONDING ANTIBODY IS ABSENT FROM THE SERUM.

What started out to be a very simple explanation of the ABO blood groups was complicated in 1923 by the discovery of two subgroups of the A antigen. Since the blood group is named by the antigens present, it meant that there are subgroups of group A and AB persons. These subgroups are clinically important and group A and AB individuals are clinically recognized as being A_1, A_2 or A_1B or A_2B. This has been further complicated by the discovery of A_3, A_4, A_5, and A_x.

ABO BLOOD GROUPING:

To determine the ABO blood group of a blood sample at least two separate tests must be done. First, the ABO group is determined by using known antibodies to identify unknown antigens. This is termed "forward grouping" or "cell grouping," and may be accomplished by either a slide test or a test tube method. Secondly, the ABO group must be confirmed, or proven, by using known antigens (known A_1 and B cells) in search of unknown antibodies. This is known as "serum grouping" or "confirmation testing" and can only be tested by the test tube method. Thus to group whole blood correctly, we determine the ABO group (antigens present) by employing known antisera and confirm these findings by identifying the antibodies present using known cells.

Cell Grouping

Cell grouping may be accomplished using either the slide method or the tube technique. The slide method is considered less sensitive than the test tube method. Both methods are based on the principle that if cells of unknown antigenic content are placed in contact with antisera containing known specific antibodies, agglutination will take place if antigens specific for the antibodies are present. If agglutination does not occur, antigens specific to the antibody are not present.

Slide Method

1. Materials necessary.
    - a. Clean glass microscope slides.
    - b. Wooden applicator sticks.

2. Reagents necessary.

a. Anti-A grouping serum.

b. Anti-B grouping serum.

NOTE: These grouping sera must meet the standards of the National Institute of Health (NIH) regarding titer, avidity, and specificity.

3. Procedure.

a. Divide a glass microscope slide in half by marking through the center with an indelible marker. Mark the left side "A" and the right side "B". Label the slide with the identifying number or name of the blood being tested.

b. Place one drop of anti-A serum in the square marked "A" and one drop of anti-B in the square marked "B".

c. With the aid of a wooden applicator stick, transfer a small amount of blood to each side of the slide. The amount of blood, regardless of source, should not exceed one half the amount of antisera present.

d. Mix both sides using separate applicator sticks. Mixing should be in a circular manner spreading the serum-cell mixture into a circle slightly larger than a nickel.

e. Observe for agglutination while tilting the slide gently so that the cell-serum mixture gathers in drops at the lowest part of the circles, and rotate gently. A white background will enhance reading.

f. Agglutination will occur within a few seconds if potent antisera are used. However, the subgroups of A may be weak and delayed, so the slide should be observed for at least 2 minutes. Thereafter, the test is considered negative for agglutination.

Table II

Interpretation of Cell Grouping

Reaction to Anti-A Serum	Reaction to Anti-B Serum	Cell Group
Agglutination (+)	No Agglutination (-)	Group A
No Agglutination (-)	Agglutination (+)	Group B
Agglutination (+)	Agglutination (+)	Group AB
No Agglutination (-)	No Agglutination (-)	Group O

4. General Considerations for Cell Grouping by Slide Method.

a. If there is any doubt concerning interpretation, repeat the test employing the test tube method.

b. Do not heat or incubate above room temperature. The ABO antigen-antibody reaction optimum temperature is 4° to 6°C. Heating may weaken or reverse the reaction. It is routinely performed at room temperature.

Test Tube Method for Cell Grouping

1. Materials necessary.

a. Clean glass test tubes, 10 x 75 mm.

b. Wooden applicator sticks.

c. Serofuge.

2. Reagents necessary.

a. Anti-A grouping serum.

b. Anti-B grouping serum.

c. Anti-A, B grouping serum.

NOTE: These grouping sera must meet the standards of the National Institute of Health (NIH) regarding titer, avidity, and specificity.

3. Procedure.

a. With an indelible marker, mark one test tube "A" and another as "B". Label both tubes with the patient's name or identifying number.

b. Place 2 drops of anti-A grouping serum in the tube labeled "A" and 2 drops of Anti-B grouping serum in the tube labeled "B".

c. The blood to be tested may be added in one of two ways.

(1) Using two separate applicator sticks, one at a time, dip the stick tip into the blood specimen approximately 1/8th of an inch. A small amount of blood will adhere to the tip of the applicator. Transfer this blood to the tubes labeled "A" and "B" by sticking the applicator into the test tube, completely to the bottom. Gently mix the tube so that cells from the applicator stick tip go into suspension in the antisera. When the antisera has been clouded with red cells, remove the applicator stick.

(2) (Alternate) Add 2 drops of saline suspended cells (4 to 6% in 0.85% sodium chloride) to each tube and mix thoroughly.

d. Centrifuge for 15 seconds in a Serofuge.

e. Gently dislodge the "button" of cells and macroscopically observe for agglutination.

f. Results may be interpreted by the use of Table II.

g. Results interpreted as group "O" must be verified by the use of Anti-A, B grouping serum. Anti-A, B is a potent serum obtained from group O persons, and it will agglutinate all red cells except those of group O. It is especially useful in detecting weakly reacting variants of A. The procedure is the same as for grouping with Anti-A and Anti-B. Agglutination with Anti-A, B indicates that the blood specimen is not group O. Therefore, it should be retested with Anti-A and Anti-B.

4. General considerations for cell grouping by test tube method.

a. If there is any doubt concerning results, repeat the test completely and incubate the tubes at 4° to 6°C (refrigerator temperature) for 10 minutes prior to centrifugation and reading (do not freeze!).

b. Confirm all group "O" results with Anti-A, B.

c. Do not heat or incubate above room temperature.

ABO Confirmation Testing

(Also known as Serum Grouping, Reverse Grouping, or Back Grouping)

The ABO blood group is obtained by testing the red blood cells with known antibodies to detect the antigens present. To erase any doubt, the serum is tested with known A_1 and B cells to check for the presence of anti-A and anti-B. Therefore, in confirmation testing we use known antigens (agglutinogens on the A_1 and B cells) to detect the presence of unknown antibodies (agglutinins in the serum). Confirmation testing uses Landsteiner's Rule to verify the ABO group obtained through cell grouping.

1. Materials necessary.

a. Clean glass test tubes, 10 x 75 mm.

b. Serofuge.

2. Reagents necessary.

a. Group A_1 cells (known A_1 cells, or a pool of at least 5 group A bloods).

b. Group B cells.

NOTE: Known cell suspensions are made fresh daily. The concentration should be 4 to 6 percent in 0.85 percent sodium chloride.

3. Procedure.

a. Label one test tube "A" and another "B". Be sure to mark both tubes with the patient's name or the specimen number.

b. Place 2 drops of the serum to be tested in each tube.

c. Add 2 drops of the known A_1 cell suspension (4-6 percent) to the tube marked "A", and 2 drops of the known B cell suspension (4-6 percent) to the tube labeled "B".

c. Mix well by shaking.

e. Centrifuge in a Serofuge for 15 seconds.

f. Gently dislodge the cell "button" and observe for agglutination.

g. Interpret results using table III.

Table III

Interpretation of Confirmation Testing

Reaction with Known A_1 Cells and serum	Reaction with Known B Cells and serum	Antibody Present in serum	Blood Group (Back Type)
No Agglutination (-)	Agglutination (+)	Anti-B	Group A
Agglutination (+)	No Agglutination (-)	Anti-A	Group B
No Agglutination (-)	No Agglutination (-)	None	Group AB
Agglutination (+)	Agglutination (+)	Anti-A and Anti-B	Group O

4. General considerations for Confirmation Testing

a. The slide method should never be used because the unknown serum being tested may not be of a sufficient titer to demonstrate a visible agglutination.

b. Avoid hard shaking in reading because it may break up agglutination and lead to a false negative conclusion.

c. Never heat specimen above room temperature.

d. If there is any doubt, repeat the test and incubate at 4° to 6°C for 10 minutes prior to centrifugation (do not freeze!).

Discussion of ABO Cell Grouping and Confirmation Testing

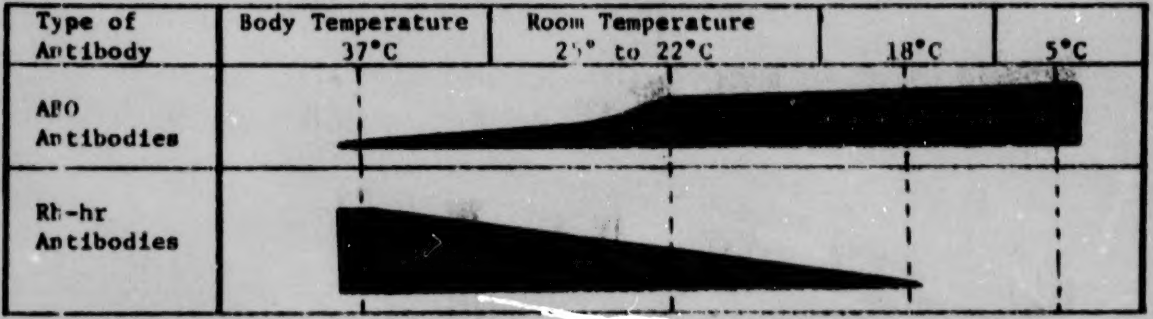
Always follow the manufacturer's directions concerning the use of ABO grouping sera. Do not cross-contaminate grouping sera. Always observe expiration dates.

Always use fresh cell suspensions prepared from recently drawn blood. The cell suspension should be used within 5 hours after preparation.

The most common error in blood grouping is incorrect labeling of tubes!

Table IV

Optimum Temperatures for Antibody Reactions



NOTE: ABO antibodies increase in reactivity as the temperature declines, having an optimum of 4° to 6°C, while Rh-hr antibodies increase in activity as the temperature increases, having an optimum of 37°C.

Table V

Optimum Medium for Antibody Reactions

Type of Antibody	Environmental MEDIUM			Complement Required
	Saline	High Protein	AHG	
ABO (Natural	+	+/-	-	No
ABO (Immune)	-	+	+	Yes
Rh-hr	-	+	+	No

THE Rh SYSTEM OF ANTIGENS

It had been suspected for many years that the cause of numerous transfusion reactions were the results of antigens other than those of the ABO blood groups. Finally, in 1940 Landsteiner and Wiener recognized another factor to which they applied the term "Rh." The factor derives its name from the original experiments with Rhesus monkeys. It was demonstrated that if red blood cells from the Rhesus monkey were injected into rabbits, an antibody (antiserum) resulted which was capable of agglutinating the Rhesus monkey erythrocytes. In addition, this antibody agglutinated human erythrocytes of 85 percent of caucasian individuals tested. Today, all commercially prepared Rh typing serum is obtained from sensitized human donors.

The Rh System Nomenclature

The Rh system differs from the ABO system in that it has no naturally occurring antibodies, and it reacts better in a high protein medium at body temperature rather than in saline at room temperature. The term Rh refers in its broadest sense to five different genetic factors (antigens) and many antigenic variants of these factors. The apparent exception to this theory is the antigen d, the existence of which has not been proven.

Two systems of terminology are currently used to distinguish these five antigens. There are the Rh-hr system of Wiener (American) and the CDE system of Fisher-Race (British). Essentially there is little difference between the two systems, but many workers find the alphabetical system of Fisher-Race more convenient to use. It is important to know both systems, since texts and manufacturers labels will use one or the other or both indiscriminately.

Table VI

Systems of Rh Nomenclature

Nomenclature of Antigens by the Wiener System (American)		Nomenclature of Antigens by Fisher-Race System (English)	
THE Rh-hr SYSTEM	Rh_o	D	THE CDE SYSTEM
	rh'	C	
	rh"	E	
	hr'	c	
	hr"	e	

Of these six antigens, the Rh_o (D) is the most important clinically. Persons are typed as either "Rh positive" or "Rh negative" strictly on the possession of this antigen. Persons having the Rh_o (D) antigen on their red cells are termed Rh positive, and those lacking it are termed as Rh negative. Approximately 85 percent of the caucasian population is Rh positive and approximately 15 percent are Rh negative.

Rh Antigen Variants

The most important variant clinically is D^u. Routine anti-Rh_o (anti-D) testing will not visibly agglutinate these cells. Therefore, anti-D testing will show these individuals to be Rh negative, while in reality they are Rh positive. The D^u may elicit the same antibody response in the Rh negative person as does the Rh_o (D). Special laboratory procedures are necessary to detect such variants (see Indirect AHG Tests).

Rh Antibodies

Extremely rarely are the Rh antibodies naturally occurring. Therefore, individuals will only demonstrate anti-Rh antibodies after improper transufsion, pregnancy, or voluntary sensitization. When an individual becomes Rh sensitized he may develop any one or all three, or the following anti-Rh antibodies:

1. Saline agglutinins (saline reactive) have been so designated because of their reactive properties; e.g., these antibodies react well both in saline and a high protein (albumin) medium. Saline agglutinins react best at a temperature of 5° to 20°C.

2. Albumin reactive agglutinins have been so designated because of the characteristics which distinguish them from saline reactive agglutinins; i.e., these agglutinins can only be detected in a high protein medium, or by the indirect AHG (Coomb's) test. Antibodies of this group react best at normal body temperature, 37°C.

3. AHG reactive Rh antibodies. These agglutinins can only be detected by the application of an indirect antihuman glubulin test (indirect Coombs).

Slide Test Method of Rh Typing

All the Rh antibodies react best at normal body temperature. The most rapid and complete clumping of the cells is obtained at 37° to 39°C. In order to provide this temperature, the surface of a viewing box must be 45° to 50°C.

1. Equipment necessary.

   a. Clean glass slides.

b. Wooden applicator sticks.

c. Light box (viewing box).

2. Reagents necessary: Anti-Rh_0 (Anti-D) typing serum that meets the NIH standards regarding specificity, titer, and avidity.

3. Procedure.

a. Using a clean glass slide, draw a large ring in the center using an indelible marker. Label the slide with the patient's name or identifying number.

b. Place 2 drops of Anti-Rh_0 (Anti-D) typing serum in the center of the ring drawn on the slide.

c. With the aid of a wooden applicator stick, add enough blood to make approximately a 40 to 50 percent cell-serum mixture.

d. Mix the serum-cell mixture with the tip of a clean applicator stick. Mix in a circular manner, spreading the solution out to cover the entire ring on the slide.

e. Place the slide on the preheated viewing box.

f. Tilt the view box back and forth and observe for agglutination.

g. Observe for a 2 minute period. Less than 2 minutes will not allow for optimum conditions to develop. Over 2 minutes will lead to drying of the cell-serum mixture; yielding false positive results.

h. Agglutination indicates that the cells being tested possess the Rh_0 (D) antigen; thus they are considered Rh positive. No agglutination indicates that the cells are Rh negative.

4. General considerations.

a. Doubtful results should be repeated employing a tube test.

b. All negative conclusions must be verified by testing for the D^u variant.

c. Excessive concentrations of anticoagulants (especially potassium oxalate) will interfere with the antigen-antibody reaction.

Test Tube Method of Rh Typing

1. Equipment necessary.

a. Clean glass test tubes, 10 x 75 mm.

b. Wooden applicator sticks.

c. Serofuge.

2. Reagents necessary: Anti-Rh$_o$ (Anti-D) typing serum that meets the NIH standards for specificity, titer, and avidity, and 22 percent Bovine Albumin.

3. Procedure.

a. Label 2 tubes with the patient's name or specimen number. Mark one tube "Rh" and the other "Control."

b. Add 2 drops of Anti-Rh$_o$ (Anti-D) typing serum to the tube marked "Rh." Add 2 drops of 22 percent Bovine Albumin to the tube marked "Control."

c. Using two separate applicator sticks, add the test cells to each tube. This may be accomplished by dipping the tip of an applicator stick approximately 1/8th inch into the sample and then immersing the cell-coated applicator in the reagent already placed in the tube. Mix well and remove the applicator stick.

d. Centrifuge in a Serofuge for 15 seconds.

e. Gently dislodge the red cell "button" and observe for agglutination. Do the same to the "Control" tube.

f. If no agglutination is seen, mix the specimen well and place into a 37°C incubator for 15 minutes. Incubate the "Control" also.

g. After incubation, spin both tubes for 15 seconds in a Serofuge.

h. Read for agglutination.

4. Interpretation

a. Agglutination indicates that the blood has the Rh$_o$ (D) antigen on the red cells. Therefore, the specimen is Rh Positive. If no agglutination is seen, the test is presumptively negative.

b. All presumptively negative tests are converted to the indirect antihuman globulin (Coomb's) test for the detection of the weakly reacting

D^u factor. If the D^u test is negative, the blood may be designated as Rh Negative (D^u Negative).

5. General considerations.

a. If results are doubtful, repeat the test. Make certain that the manufacturer's instructions are closely adhered to.

b. Agglutination in the control tube in any phase of the test automatically invalidates the results. There should never be agglutination in the control tube. If you do observe agglutination in the control tube, repeat the test employing washed packed cells.

c. All positive tests for D^u are considered as Rh Positive.

ANTIHUMAN GLOBULIN TESTS (AHG Tests):

Antihuman globulin is usually prepared from rabbits that have been immunized against human globulin. The serum is known by various synonyms such as "antihuman serum," Coomb's serum, antiglobin serum, and Coomb's test serum. AHG sera are used in the Coomb's test or antihuman globulin tests. These tests are nonspecific in that they will detect the presence of an antibody (agglutinin) coating red cells, but will not specifically identify the particular antibody (agglutinin).

The antihuman globulin test (or Coomb's Test) is used extensively in prenatal cases to detect circulating antibodies. Such antibodies may have arisen through sensitization of the mother either as a result of pregnancy or transfusion. These antibodies in the mother's serum are capable of crossing the placental barrier in some manner and coating the infant's erythrocytes. These tests may either be run qualitatively or quantitatively.

Another very important use of the AHG test is to detect immune antibodies in the serum of the recipient which might otherwise go undetected in the routine crossmatch. The presence of such antibodies could result in a hemolytic transfusion reaction.

The Direct Antihuman Globulin (Coomb's) Test

This test is employed when red cells are suspected of having been coated in vivo. Clinical suspicion of hemolytic disease of the newborn (HDN), hemolytic anemia, or a hemolytic transfusion reaction warrants that this test be performed. Coated cells when exposed to antihuman globulin will agglutinate by virtue of the attached antibodies present on the erythrocyte surface. The direct AHG test is an excellent screening procedure.

1. Equipment necessary.

   a. Clean glass test tubes, 10 x 75 mm.

   b. Serofuge.

   c. Wooden applicator stocks.

2. Reagents necessary.

   a. Antihuman globulin serum (Coomb's Serum).

   b. Normal saline (0.85 percent sodium chloride).

3. Procedure.

a. Prepare a 4 to 6 percent cell suspension in 0.85 percent NaCl of the cells to be tested. Label the tube with the patient's name or the specimen number.

b. Label 3 clean test tubes with the patient's name or sample number. Mark one tube as "Test," one as "Positive Control" and the final tube as "Negative Control."

c. Add 2 drops of the unknown cell suspension to be tested to the tube marked "Test." Add 2 drops of known AHG negative cells to the "negative control" tube and 2 drops of known AHG positive cells to the "positive control" tube. (These controls should be in a 4 to 5 percent saline cell suspension.)

d. Fill all tubes approximately three-fourths full with fresh 0.85 percent NaCl. Mix by inversion using Parafilm and Serofuge for one minute. Decant the supernatant and repeat the washing process at least four times. After the last washing, decant the supernatant and absorb all the remaining saline by blotting the lip of the tube with gauze or Kimwipes.

e. Add one drop of AHG serum to each tube.

f. Mix thoroughly and Serofuge for 15 seconds.

g. Gently dislodge the red cell "button" and observe for agglutination in each of the three tubes.

h. Interpretation

(1) Agglutination should be observed in the positive control tube. This indicates that the method and reagents are functional. If no agglutination appears, the AHG serum is bad or a procedural error has been made.

(2) No agglutination should be observed in the negative control tube. This insures that there is no pseudoagglutination present, and further insures procedural adherence. Agglutination would indicate a technical error or faulty reagent.

(3) Agglutination in the "Test" sample indicates that the red cells have been sensitized by an antibody in vivo. This conclusion cannot be made if the controls used do not conform as outlined in (1) and (2), above.

The Indirect Antihuman Globulin (Coomb's) Test

This test is employed to detect antibodies by causing them to coat red cells in vitro. It is particularly useful in prenatal studies because the test will detect circulating antibodies in the mother's serum. The test is also used to determine the presence of immune antibodies in the recipient's or donor's serum during blood crossmatching. A modification of this test is used to demonstrate the D^{u} factor when confirming persons as Rh negative. (Demonstration of an unknown antigen through use of a known antibody.)

Erythrocytes of a known type (antigenic content) are exposed, in vitro, to the serum to be tested (unknown antibodies) to determine if the latter contains antibodies capable of coating these cells. The test is continued at this point as in the direct method. Thus, agglutination indicates the presence of circulating antibodies in the patients' serum and no agglutination indicates a lack of circulating antibodies.

The test red cells used in this procedure should have as many antigens as possible on their surface to detect as many different antibodies as possible. But, they should have no ABO antigens so that agglutination would not occur from the naturally-occurring antibodies. Thus, all screening cells used in indirect AHG (Coomb's) testing should be ABO group "O" (having no A or B antigens).

1. Equipment necessary.

    a. Clean glass test tubes, 10 x 75 mm.

    b. Wooden applicator sticks.

    c. A 37°C incubator.

    d. Serofuge.

2. Reagents necessary.

    a. Antihuman globulin serum (Coomb's serum).

b. Group "O" test cells of known antigen content.

c. Normal saline (0.85 percent sodium chloride).

3. Procedure.

a. Prepare a 4 to 6 percent cell suspension of the test cells in fresh normal saline.

b. Label three clean test tubes with the patient's name or sample number. Mark one tube "Test," one as "Positive Control" and the last as "Negative Control."

c. To the tube marked "Test" add two drops of the serum to be tested. To the positive control add 2 drops of a serum known to contain an antibody, and add 2 drops of a serum known to be antibody-free to the negative control tube.

d. Add 2 drops of the known screening cell suspension to all three tubes and mix.

e. Incubate all tubes for 15 minutes at 37°C. This allows any antibodies present to coat the reagent cells.

f. After incubation, wash the cells four times and apply AHG serum as outlined in the direct procedure (steps d, e, f, and g).

g. Interpretation.

(1) Agglutination should appear in the "Positive Control" tube, thus insuring that the test system and reagents are sound.

(2) No agglutination should be observed in the "Negative Control" tube, thus ruling out false agglutination in the test system.

(3) Agglutination in the tube containing the sample ("Test") indicates that an antibody (or antibodies) has reacted with the antigen (or antigens) on the test red blood cells, hence the patient is proven to have a circulating antibody. No agglutination shows that the patient lacks any detectable circulating antibodies. (Results valid only if controls are normal.)

THE CROSSMATCH:

Before a transfusion is performed, both the donor and recipient's blood must be checked for ABO group and Rh type. Only like ABO and Rh bloods should be considered for transfusion and only then if the bloods are matched against each other and found to be compatible.

The crossmatch not only checks the compatibility of each specific unit of blood with the blood of the recipient, but also serves to recheck the original group. In the crossmatch, the donor's red cells are exposed to the recipient's serum (Major Side) and the recipient's red cells are exposed to the donor's serum (Minor Side) to see if any antigen-antibody reaction takes place. Agglutination on either side indicates incompatibility between the donor's blood and that of the recipient. Thus, the donor unit of blood is considered incompatible for this particular transfusion.

This exposure of the antigens of the donor (his red cells) to the antibodies of the recipient (his serum) and vice versa, is performed in vitro. Thus, in vivo conditions must be simulated as close as possible. Also, the optimum conditions for a great number of possible antigen-antibody reactions must be provided. These conditions and requirements are met by the two tube, three phase crossmatch procedure.

1. Equipment necessary.

    a. Clean glass test tubes, 10 x 75 mm.

    b. Wooden applicator sticks.

    c. Transfer pipets.

    d. 37°C incubator.

    e. Serofuge.

2. Reagents necessary.

    a. Antihuman globulin serum (Coomb's serum).

    b. Normal saline, 0.85 percent sodium chloride.

    c. Bovine albumin reagent (22 to 30 percent).

3. Procedure for the 2 tube, 3 phase method.

NOTE: In conjunction with this procedure, testing of the donor's blood sample and the recipient's blood sample is performed. These tests include ABO cell and serum grouping by tube method, tube method Rh tests that include D^u testing when indicated. Also, donor and recipient sample verification of identity must be made. These procedures are outlined elsewhere in this manual and will not be repeated here.

a. With an indelible marker, label two tubes with the patient's name or number. Mark one tube "Major Side" and the other "Minor Side."

b. Add saline suspended cells (4 to 6 percent) and serum as follows:

MAJOR SIDE TUBE	MINOR SIDE TUBE
Donor's Cells - 2 drops	Recipient's Cells - 2 drops
Recipient's Serum - 2 drops	Donor's Serum - 2 drops

c. Mix gently and Serofuge for 15 seconds.

d. Examine both tubes for agglutination (and/or hemolysis). If either the "Major" or "Minor" tube shows agglutination, stop. The crossmatch can be termed incompatible. If no agglutination, continue with the following steps.

NOTE: This completes phase I - the saline phase at room temperature. Any antigen-antibody reactions that react best in a saline medium and cold temperature should be demonstrated during this phase.

e. Add 2 drops of bovine albumin to each tube; mix gently. This step provides a high protein environment for those reactions that require or are enhanced by a high protein medium.

f. Serofuge for 15 seconds and observe for agglutination. Positive agglutination in either tube is evidence of incompatibility and the test may be stopped. If no agglutination is seen, continue to next step.

g. Incubate both tube at 37°C for a minimum of 15 minutes.

h. Centrifuge in Serofuge for 15 seconds.

i. Examine for agglutination. Positive agglutination in either tube constitutes incompatible crossmatch and you may stop. If there is no agglutination or hemolysis, continue with the procedure.

NOTE: This completes the high protein phase, or albumin phase, at both a cold temperature and normal body temperature. Antigen-antibody reactions occurring optimally in a high protein environment should be detected during this phase.

j. Wash both tubes with normal saline a minimum of four times.

k. Add one drop of AHG serum to each tube and mix gently.

l. Serofuge for 15 seconds.

m. Examine visually and microscopically for agglutination. No agglutination is considered compatible, while agglutination is cause for rejection.

NOTE: This completes Phase III, or AHG phase. All reactions best detected by a Coomb's procedure should be detected during this phase.

4. General Considerations and Precautions.

a. Should the cell-serum mixtures survive this test, it can be assumed that neither donor nor recipient possess antigens that have a corresponding antibody in the serum of the other individual. Therefore, the blood should be safe for transfusion.

b. Always use serum rather than plasma.

c. Label all tubes correctly and indelibly.

d. In washing cells, never use your finger over the end of the tube to mix it. Watch for other forms of contamination to the AHG test system by human protein from your hands.

e. Causes of false compatible crossmatches:

(1) Errors in which the cells and serum from the same person are inadvertently combined.

(2) Excessive shaking when reading for agglutination.

(3) Insufficient cell washing by inadequate saline volume, inadequate draining, contamination with human hands, or insufficient number of washes.

(4) Improper incubation periods.

(5) Improper cell concentrations.

(6) Outdated or contaminated AHG serum.

(7) Specimen samples that are not fresh.

f. Causes of false incompatible crossmatches:

(1) Rouleaux formation, or drying of serum-cell mixtures.

(2) Excessive centrifugation.

(3) Hemolysis due to rough handling rather than from the antigen-antibody reaction.

(4) Nonspecific agglutination due to chemical contamination of the glassware, or bacterial contamination of the serum or cells.

(5) Nonspecific agglutinins.

(6) Coagulation due to excessive heat.

g. Human error, technical or administrative, is by far the greatest source of error in blood banking and crossmatching.

TESTING FOR Du FACTOR:

Blood which is D^u positive will appear to be type D negative when routine Rh testing procedures are employed. For this reason, AHG serum (Coomb's Serum) must be used to demonstrate the presence of the D^u factor.

1. Equipment necessary.

   a. Clean glass test tubes, 10 x 75 mm.

   b. Serofuge and 37°C incubator.

   c. Transfer pipets.

2. Reagents necessary.

   a. Antihuman globulin serum (Coomb's serum).

   b. Normal saline (0.85 percent sodium chloride).

3. Procedure.

   a. When an Rh tube test has been performed along with a control and both the "test" and the control show no agglutination a presumptive interpretation of Rh negative is made. The D^u test is then called for and set up according to manufacturer's directions. The "test" tube contains the unknown cells and anti-D (anti Rh_o) typing serum and the control tube, the unknown cells, and bovine albumin.

   b. Incubate at 37°C for 15 minutes.

   c. Serofuge for 15 seconds.

d. Mix gently and observe for agglutination. If there should be agglutination, the test is considered Rh positive and just a weakly reacting D positive. If negative (which it will usually be) continue the D^u test.

e. Wash both tubes at least four times.

f. Add one drop of AHG serum to each tube and mix gently.

g. Serofuge for 15 seconds and read for agglutination.

h. Agglutination in the "Test" tube indicates a D^u positive, while no agglutination means a true Rh negative. The control tube should be negative for agglutination. If the control is positive all results and conclusions are invalid.

GLOSSARY OF TERMS:

In order to fully understand the basic reactions which take place in blood banking, a knowledge of certain terms and facts about immunohematology must be known.

A.A.B.B. (American Association of Blood Banks): Prescribes minimum procedural standards for blood banks, both military and civilian.

AGGLUTINATION: Collection into clumps of the blood cells or particles distributed in a serum or other fluid.

AGGLUTININS: Antibodies which cause agglutination of cells in a serum or other fluid suspension.

AGGLUTINOGEN: A receptor site (antigen site) on the surface of a blood cell which, in the presence of its opposing antibody, produces agglutination of the blood cells.

ANTIBODIES: A specific substance, usually protein in nature (globulin), produced by and in an animal in response to a provoking antigen. There are several types of globulins with antibody activity, but they are known collectively as immunoglobulins. All agglutinins are antibodies but not all antibodies are agglutinins.

ANTIGEN: Any foreign substance capable of provoking an immune response (production of an antibody) in an animal capable of antibody production. All agglutinogens are antigens, but not all antigens are agglutinogens.

ANTIHUMAN GLOBULIN (Anti-globulin): An antibody produced in an animal (usually a rabbit) in response to an injection (antigenic stimulation) of human globulins.

ANTI-SERUM: Serum from an individual immunized against a specific antigen, and therefore containing antibodies specific for that antigen.

AVIDITY: The time (in seconds) it takes for an antiserum to agglutinate a suspension of cells possessing opposing agglutinogens (antigens).

BLOOD FACTOR: A serologic factor occurring on the surface of an erythrocyte; e.g., the blood factors A, B, Rh_o, etc.

BLOOD GROUP: The classification of blood on the basis of the antigens present on the red cell surface. The process of performing this is known as blood grouping.

BLOOD GROUP SYSTEM: A system of related blood group factors and antigens; e.g., the ABO blood group system, the Rh-hr system, the MNS system, etc.

BLOOD GROUPING: Classification of blood specimens into groups on the basis of the blood factors or agglutinogens contained on the red cell surfaces.

CHROMOSOME: One of the dark staining, rod-shaped bodies appearing within the nucleus of a cell at the time of cell division. It contains the inheritance factors or genes.

CO-DOMINANT GENES: Genes which are expressed equally well on the red cell surface. All immunohematological blood group factors of medical importance serve as examples of co-dominance.

COMPATIBLE BLOOD: Blood which has been tested *in vitro* against the recipient's blood and shows no visible reaction.

COMPATIBILITY TEST: A test for serologic compatibility carried out between a patient's serum and the donor's red cells, and the donor's serum and the patient's red cells.

COMPLEMENT: A series of protein substances in normal serum which cause lysis of red cells sensitized by hemolytic antibodies.

CONTROL: A test system used to verify the correctness of testing observations. Usually set up to check reagents, techniques, and environmental conditions in the laboratory.

COOMB'S TEST: A test using antihuman globulin to detect antibodies (which are globulins) coating red cells.

CROSSMATCH: (See compatibility test.)

DIRECT ANTIHUMAN GLOBULIN TEST (Direct Coomb's Test): A test for the sensitization of red cells in vivo, by the use of Antihuman Globulin Serum (Coomb's serum).

DOMINANT GENE: A gene which suppresses the action of another gene of its pair group, if present in a heterozygous condition. The other gene is said to be recessive.

ERYTHROBLASTOSIS FETALIS (Hemolytic Disease of the Newborn): A disease of infants caused by an antibody produced in the mother in response to a paternal antigen on the red cells of the infant.

FISHER NOMENCLATURE SYSTEM (Fisher-Race System): A system of nomenclature in which the Rh-hr antigens are named by the symbols C, D, E, c, e, (d?).

GENE: The basic unit of inheritance. Each gene carries one unit of inheritance (transmissable characteristic) on the chromosome and is arranged with its fellow genes in a linear order.

GENOTYPE: The fundamental hereditary structure. The genetic makeup for a particular characteristic carried by an individual. The sum total of the genes present to produce an individual characteristic.

GLOBULIN: A serum protein. All antibodies are globulins.

HEMOLYSIN: An antibody which, under the proper conditions, will cause the lysis of red cells.

HEMOLYSIS: The rupture of red blood cells with the release of hemoglobin, caused by changes in the cell permeability in the presence of complement.

HEMOLYTIC ANEMIA: A condition characterized by low red cell counts, caused by a shortened life-span of these cells within an individual.

HEMOLYTIC DISEASE OF THE NEWBORN (HDN): (See erythroblastosis fetalis.)

HETEROZYGOUS: The state in which allelic genes on the corresponding loci of a pair of chromosomes are unlike (unlike genes).

HOMOZYGOUS: The state in which allelic genes on the corresponding loci of a pair of chromosomes are alike (like genes).

IMMUNIZATION: The introduction of foreign antigens by such means as a heterospecific pregnancy, an incompatible blood transfusion, or an injection. An immunization is characterized by the production of an antibody response.

INCOMPATIBILITY: Agglutination (or lysis) of cells due to an antigen-antibody reaction. In immunohematology it usually refers to a reaction seen in vitro in a crossmatch.

INCUBATE: To maintain a cell-serum mixture at a specific temperature for a specified period of time in order to obtain maximum reactivity.

INDIRECT ANTIHUMAN GLOBULIN TEST (Indirect Coomb's Test): A test used to determine the presence of a given antibody and its corresponding antigen site on red cells. A test to detect in vitro antibody coating of red cells by the use of antihuman globulin serum (Coomb's serum).

IN VITRO: An in vitro reaction occurs outside the body; e.g., in a test tube.

IN VIVO: An in vivo reaction occurs within a living organism (in the body).

ISOIMMUNIZATION (isosensitization): A process, accidental or otherwise, whereby an individual's system is stimulated to produce a specific antibody (which it formerly lacked) to a foreign red blood cell of the same species.

LOCUS: The point on a chromosome occupied by a given gene.

LYSIS: A term for loss of hemoglobin from red blood cells due to changes in the cell wall permeability due to an antibody and complement.

MAJOR SIDE CROSSMATCH: A term for an in vitro test between the donor's red blood cells and the recipient's serum.

MINOR SIDE CROSSMATCH: A term for an in vitro test between the recipient's red blood cells and the donor's serum.

NATURAL ANTIBODIES: Antibodies normally present with members of a given animal species; e.g., anti-A and anti-B in the human species.

N.I.H.: The National Institutes of Health. The Division of Biologic Standards, NIH, is responsible for setting minimum standards for antiserum to be used in immunohematology.

NONSPECIFIC COLD AGGLUTININ: An antibody (or antibody-like material) which usually causes agglutination of red cells at low temperatures, but which disappears when warmed.

PHENOTYPE: The observable characteristics of an individual.

PLASMA: The liquid portion of anti-coagulated blood.

POOLED CELLS: A suspension of cells derived from several different donors.

REACTION: The term used in immunohematology to indicate a visible agglutination, lysis, or neutralization of cells by a serum.

REAGENT RED BLOOD CELLS (HUMAN): Sometimes known as 0 screening cells. Erythrocytes of known antigen content, usually used for antibody detection and/or identification.

ROULEAUX FORMATION: A cell-stacking effect resembling a stack of coins, often caused by drying of the sample, which may cause false positive agglutination readings by inexperienced technicians.

SENSITIZED RED CELLS: Red cells coated with an antibody.

SENSITIZATION: (See Immunization.)

SPECIFICITY: The special affinity of an antigen only for its corresponding antibody.

TITER: The highest dilution of a serum which yields a visible reaction under appropriate reaction conditions.

WASHED CELLS: Cells which have been freed of plasma and other contaminants by repeated washing and centrifugation with large volumes of normal saline.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-119
LABORATORY SCIENCES DIVISION 075
CLINICAL MICROSCOPY BRANCH

Practical Examination and Critique II (14 hours)

COURSES PRESENTED TO: 311-92B20 Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES:

TM 8-227-3

STUDY ASSIGNMENT: All previous material

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: None

PERSONNEL: One primary instructor (MOS 92B4H, 92B3H); two secondary instructors (MOS 92B4H, 92B3H)

INSTRUCTIONAL AIDS: PA system with lapel microphone, chalk, chalkboard and eraser

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Practical examination

I. INTRODUCTION (5 min)

A. Opening Statement: The tests performed prior to the actual administration of a unit of blood insure the patient's safety during transfusion therapy. It is your responsibility as the blood bank technician to perform these pre-transfusion compatibility test procedures accurately to preclude any transfusion reaction when the blood is administered to the patient. Errors in performing compatibility tests may result in serious injury or even death to the patient.

B. Objectives

Perform procedures practiced during the course with attainment of an equivalent grade of at least 70 percent.

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*This lesson plan supersedes LP 08-280-109, 014.

C. Class Procedure and Lesson Tie-in

1. Fourteen hours of practical examination determining students' ability to perform all tests and procedures previously presented.
2. Conferences after examination as necessary.

II. APPLICATION (550 min)

A. Arrangement of Students

1. Students will work at their regularly assigned area during the practical examination
2. Students will be at their regularly assigned area during the reteach session

B. Instructions to Students

1. Pick up blood specimens from instructor
2. Pick up reagents and equipment for examination from instructor
3. Check chalkboard in classroom at the beginning of each period for special instructions
4. No talking or discussion of test results
5. Report results of testing on forms provided by instructor

C. Instructors' Activities

1. Prior to class
    - a. Prepare reagents as needed
    - b. Prepare and key unknown specimens for student analysis
2. During class
    - a. Issue materials, reagents and specimens as necessary
    - b. Monitor students' performance of procedures closely, making immediate corrections
    - c. Grade completed report forms and record results immediately
    - d. Maintain classroom control

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D. Students' Activities

1. Under instructor's supervision, students will perform:

   a. ABO group and confirmation

   b. RH_o (D) determination

   c. Direct anti-human globulin test

   d. Indirect anti-human globulin test

   e. Antibody screen

   f. Compatibility test

2. Students will be responsible for maintenance of individual equipment

3. Students will record the results of daily practical examination on forms provided, and hand them in to be checked by instructor

III. REVIEW (5 min)

A. Questions From Students

B. Review of Main Points Covered During Reteach Session

C. Closing Statement: How many mistakes did you make in this practical examination? If you consider each mistake as a seriously injured or lost patient you immediately understand why these procedures must be performed without error in the clinical laboratory.

INSTRUCTIONAL HOURS

Number of instructors required - - - 3

Primary hours	14
Secondary hours	0
Total	14

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-120
LABORATORY SCIENCES DIVISION 075
CLINICAL MICROSCOPY BRANCH

Written Examination and Critique II (1 hour)

COURSES PRESENTED TO: 311-92B20 Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES:

TM 8-227-3

STUDY ASSIGNMENT: Before class, read and review all previous material

STUDENT UNIFORM AND EQUIPMENT: None

TOOLS, EQUIPMENT, AND MATERIALS: None

PERSONNEL: One primary instructor (MOS 92B4H or 92B3H)

INSTRUCTIONAL AIDS: PA system with lapel microphone, chalk, chalkboard and eracer.

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Written Examination

I. INTRODUCTION (5 min)

A. Opening Statement: The life of critically ill patients is entrusted to you when you perform pre-transfusion compatibility tests. To be considered competent in performing these crucial procedures you must have a sound understanding of the principles and theory upon which these tests are based.

B. Objectives

1. Answer questions on theories, principles, and procedures presented in lecture.

2. Demonstrate an ability to interpret the results of immunohematological test procedures given specific and example test data.

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*This lesson plan supersedes LP 08-280-120, 014.

C. Class Procedure and Lesson Tie-in

1. Final two hour of 64 hours of immunohematology.

2. Reteach and conferences, as necessary, to solve individual problems.

II. APPLICATION (70 min)

A. Arrangement of Students

1. Students will be spaced as conditons and number of students permit during written examination.

2. Students will be at their regularly assigned work area during reteach session.

B. Directions to Students

1. No written or printed material of any type will be permitted during written examination

2. No talking allowed during written examination

3. Check chalkboard at the beginning of class for special instruct

4. During reteach students are encouraged to ask questions

C. Instructors' Activities

1. Space students for written examination as conditions and number of students permit

2. Issue test booklets and answer sheets (one per each student)

3. Grade answer sheets and record results immediately

4. Answer students' questions during reteach session and reteach as indicated by examination results

5. Circulate in classroom during examination

6. Maintain classroom control

D. Students' Activities

1. Students will record answers on forms provided by the instuctor and hand this form and test booklet in to the instructor.

2. Students will be responsible for work area.

III. REVIEW (5 min)

A. Questions From Students

B. Review of Main Points Covered During Reteach Session

C. Closing Statement: The life of the patient is literally entrusted to your care when you perform many of the procedures in the Blood Bank. His recovery depends upon your skill and accuracy in performing all of these tests. If you remember the principles presented during this course you have the capacity to aid the patient's recovery through your part in transfusion therapy.

CHAPTER 4
ABO BLOOD GROUPING PROCEDURES

Blood grouping is not an end in itself but rather a means to an end. That end is to make transfusion of blood as safe as possible. This objective makes correct blood grouping of donor and recipient absolutely mandatory. It is believed that an understanding of the principles underlying test reactions will be an aid to the technician in intelligently performing the procedures. For this reason, a brief discussion pertinent to the makeup of the ABO blood system is presented here.

The four basic blood groups A, B, AB, and O were discovered by Karl Landsteiner and his coworkers in 1900 and 1902. The nomenclature for the blood groups is based upon specific antigens called agglutinogens present on the erythrocytes. Group A blood possesses A agglutinogen; group B, the B agglutinogen; group AB, both agglutinogens; and group O possesses neither agglutinogen.

Naturally occurring antibodies called agglutinins are also known to be present in human blood. These agglutinins, anti-A and anti-B, occur in the plasma or serum. The agglutinins will react with the corresponding agglutinogens, and so we find that normally they are never found together with their corresponding agglutinogen. Table 4-1 outlines the makeup of the four basic blood groups.

Table 4-1. Characteristics of the Four Basic Blood Groups

Landsteiner or international nomenclature	Agglutinogens on cells	Agglutinins in serum	Frequency in U.S. caucasians
O	Neither A nor B	Anti-A Anti-B	45%
A	A	Anti-B	40%
B	B	Anti-A	10%
AB	A and B	None	5%

A AND B AGGLUTINOGENS

The A and B agglutinogens have been demonstrated on erythrocytes in the developing embryo as early as the 40th day after conception. The agglutinogens exhibit a low degree of reactivity in the newborn but there is a gradual increase throughout postnatal life. The maximum reactivity is attained during the late teens or early twenties and remains fairly constant throughout the remainder of life. It is important to realize that the agglutinogens are always developed before birth and are inherited from the mother and father.

Approximately 80 percent of the population produces a water soluble form of their blood group substance which is present in most body fluids and in secretions such as saliva. These individuals are known as secretors.

ANTI-A AND ANTI-B AGGLUTININS

The anti-A and anti-B agglutinins appear initially during the first few months of postnatal life. An individual's agglutinin titer exhibits a gradual increase until the late teens or early twenties. In early adult life, the titer remains rather stable for approximately 10 years and then gradually decreases.

During the earlier discussion concerning basic immunology, it was stated that antibodies are produced as a result of an antigenic stimulus. The anti-A and anti-B antibodies presently being discussed represent an exception to this general rule. These antibodies are, according to present evidence, governed by genetic factors and, therefore, occur naturally. It must be remembered, however, that they do not become demonstrable until after birth.

Occasionally agglutinins may be detected in the newborn. These are usually not the infant's own antibodies but those which have passed through the placental wall from the mother. The newborn infant, then, cannot normally possess any agglutinin which the mother does not already have. These transplacental agglutinins disappear rapidly after birth, and in approximately 70 percent of the cases, are completely gone after the first 10 days of life. In rare cases, however, they may remain for several months.

INHERITANCE OF ABO BLOOD GROUPS

Within the nuclei of living cells there are dark staining rod-shaped bodies called chromosomes. Chromosomes are believed to be composed of minute units referred to as genes. A gene may be thought of as the basic unit of inheritance which carries one inheritable character from the parent to the offspring. The genes are arranged in linear fashion within the chromosomes.

The demonstrable blood group of an individual is the direct result of inheritance of the agglutinogen from one or both of his parents. The agglutinogens are influenced by three genes: A, B, and O. Genes A and B are equally dominant while gene O is considered to be recessive. That is, if gene O is present with gene A or B in the chromosomes, only the agglutinogen A or B will appear on the individual's blood cells.

Considering one pair of an individual's chromosomes, the gene pattern or genotype for the ABO blood group is composed of two genes. These may be alike or different. If they are identical, the individual is called homozygous for those genes. If the genes in the genotype are different, the individual is referred to as heterozygous.

The phenotype represents the actual blood agglutinogens which develop on the blood cells as a result of the influence of the genotype. The phenotype is detectable by employing antisera containing the corresponding specific agglutinins. Table 4-2 indicates the phenotypes and genotypes of the four basic blood groups.

Table 4-2. Phenotypes and Genotypes of the Four Basic Blood Groups

Phenotype	Genotype
O	OO
A	AA or AO
B	BB or BO
AB	AB

The genotype possessed by an individual is the result of a process which begins in the parents. In every precursor cell of the mature sperm or ovum, there are 23 pairs of chromosomes (until recently thought to be 24 pairs). Among these 46 chromosomes, there are two which possess one gene each for the blood group agglutinogens. A typical precursor cell is shown in figure 4-1. For the sake of clarity, only the 2 chromosomes involved in ABO inheritance will be represented.

As a result of the division of these precursor cells of the parents, two mature gametes (sperm or ovum) are formed. Each gamete now contains a single chromosome of the original pair. When the mature gametes (sperm and ovum) from the two parents unite, the resulting pair of genes (within the chromosomes) determine the child's genotype, figure 4-2.

The union of the gametes from the two parents occurs entirely by chance so that a parent may contribute either one of the genes from his or her genotype but not both.

The possible resulting blood groups from various matings are given in table 4-3.

Table 4-3. Possible Blood Groups Resulting from Various Parental Combinations

Parent's blood group		Possible group of children
Mother	Father	
O	O	O
O	A	O or A
O	B	O or B
O	AB	A or B
A	A	A or O
A	B	A, B, AB, or O
A	AB	A, B, or AB
B	B	B or O
B	AB	A, B, or AB
AB	AB	A, B, or AB

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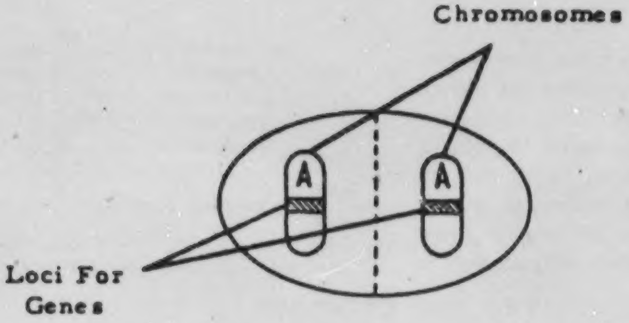

Figure 4-1. Immature reproductive cell prior to division.

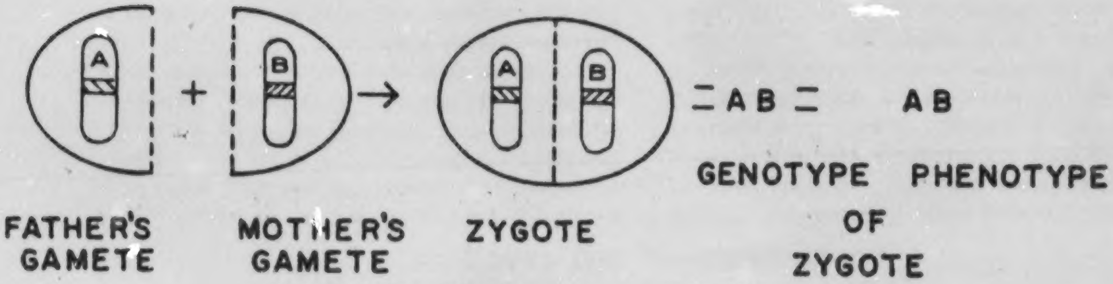

Figure 4-2. The union of gametes showing the resulting blood group.

The above information is sometimes employed in medicolegal cases where infant exchange in nurseries or disputed paternity are involved. By knowing the parents' blood groups, it is possible to predict which blood groups could be possessed by the infant. Similarly, if the blood groups of the mother and infant are known, it is possible to predict which blood group would have to be possessed by the father. Medicolegal considerations of blood groups will be presented in greater detail in chapter 9.

BLOOD GROUPING TEST REACTIONS

In blood grouping tests, the technician observes for agglutinations reactions occurring between cells possessing agglutinogens and corresponding agglutinins in a plasma or serum. These reactions will be of two types, anti-A agglutinin reacting only with A agglutinogen and/or anti-B agglutinin reacting only with B agglutinogen.

For proper determination of the ABO blood group of an individual at least two separate tests should be performed by the technician. The recommended approach for grouping blood is to first test the unknown cells with the known grouping sera meeting National Institutes of Health (Division of Biologic Standards) standards. The blood group is then confirmed by testing the unknown serum with known A and B cells. These procedures, known as serum grouping or back typing, will be considered in detail later.

ABO CELL GROUPING

Two separate sera with known agglutinins are employed for grouping unknown cells. These sera are referred to as anti-A serum and anti-B serum. Anti-A serum contains a high titer of anti-A agglutinins and anti-B serum contains a high titer of anti-B agglutinins.

The reactions encountered between the known sera and the unknown cells are given in table 4-4.

Table 4-4. Reactions Occurring During Cell Grouping

Blood cells from group	Reaction in anti-A serum	Reaction in anti-B serum
O	No clumping	No clumping.
A	Clumping	No clumping.
B	No clumping	Clumping.
AB	Clumping	Clumping.

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Cell grouping can be performed using a slide technique or a tube test. The slide technique, to be considered first, is considered less sensitive than the test tube methods and more likely to exhibit rouleaux formation. The slide technique is commonly used, however, because it requires less equipment and is more rapid and convenient. Slide tests can be run routinely and tube tests reserved for confirmation checks as well as for specimens which give doubtful slide reactions. A reaction temperature of 4° C. should be used when discrepancies occur between direct grouping and back typing.

Slide Method

The slide method of blood cell grouping may be considered the standard routine test. If performed exactly according to instructions provided by the antiserum manufacturer, it will be quite adequate for the great majority of blood specimens.

APPARATUS:

Glass microscope slides.
Wooden applicator sticks.

REAGENTS:

Anti-B grouping serum.
Anti-A grouping serum.

Note. These sera must meet the standards of the National Institutes of Health (Division of Biologic Standards) regarding potency, avidity, and specificity.

TECHNIQUE:

1. Using a wax pencil, divide a glass microscope slide in half by marking through the center.
2. Mark the left half of the slide "A" and the right half "B." Identify with the patient's name or code number.
3. Place one drop of anti-A serum in the square marked "A" and one drop of anti-B serum in the square marked "B."
4. With the aid of a wooden applicator stick and using finger blood, clotted, oxalated, or citrated whole blood, transfer a small amount of the blood specimen (not greater than one-half the volume of serum used) to the drop of anti-A serum. Using the same applicator stick employed for transferring, mix the drop of antiserum and blood. The mixing should be in a circular pattern so as to spread the cells and serum into a circle approximately 25 mm. in diameter. Using a clean applicator stick transfer a small amount of the blood specimen to the drop of anti-B serum and mix as before. Be sure to use a clean applicator stick for each side of the slide.
5. Tilt the slide so that the reaction mixtures gather in drops at the lowermost parts of the circles. Gently rotate so that the drops move along the edges of the circles at a speed of approximately six revolutions per minute. Gentle rotation and tilting is always performed as a preliminary to reading the reaction.
6. When slide tests are performed with potent antisera and whole blood, it is not necessary to observe for extended periods of time before designating a blood group. Agglutination will occur within a few seconds except in the case of weakly reacting A and AB cells. In all cases, readings should be made within 3 minutes.
7. Reading is accomplished macroscopically with the slide held against a white background. Agglutination, when present, is always obvious. (***Caution:* (Rouleaux may cause confusion.**) Ordinarily it is not necessary to observe microscopically for agglutination. In rare instances, if doubt should exist, the serum-cell mixture should be examined under the low power objective of the microscope.

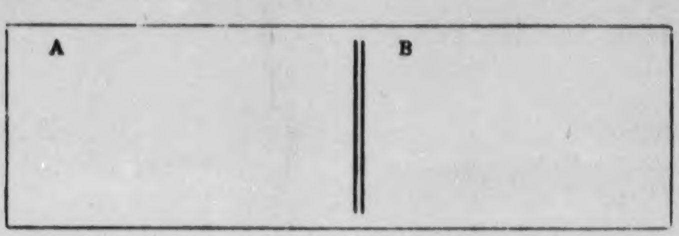
Note. Do not apply heat to the slides in this test. The action of anti-A and anti-B agglutinins is not enhanced and may be weakened or even reversed if heat is applied. This technique is summarized in table 4-5. Anti-A and anti-B react best at 4° C.

INTERPRETATION:

The blood group is determined by the presence or absence of agglutination (clumping) with anti-A and anti-B sera as indicated in table 4-6. This procedure is illustrated in figure 4-3① and 4-3②.

Table 4-5. Slide Method of Blood Cell Grouping

Slide divided in half and labeled



Anti-A grouping serum	**One drop**	
Anti-B grouping serum		**One drop.**
Blood specimen (whole blood)	**Cells adhering to applicator stick.**	**Cells adhering to applicator stick.**

Mix with separate applicator sticks. Tilt and rotate slide. Observe for clumping within 3 minutes.

REACTION		
Group O	**No clumping**	**No clumping.**
Group A	**Clumping**	**No clumping.**
Group B	**No clumping**	**Clumping.**
Group AB	**Clumping**	**Clumping.**

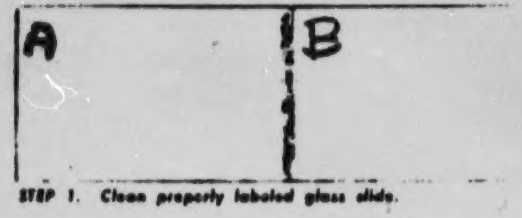
NOTE: Known A and B cells should be tested simultaneously as controls.

Table 4-6. Interpretation of Blood Grouping Reactions

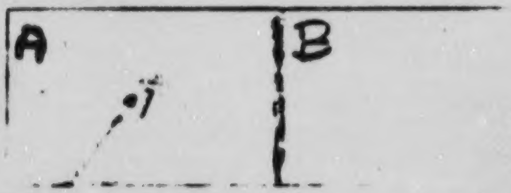
Blood cells from group	Reaction in anti-A serum	Reaction in anti-B serum
O	No clumping	No clumping.
A	Clumping	No clumping.
B	No clumping	Clumping.
AB	Clumping	Clumping.

DISCUSSION:

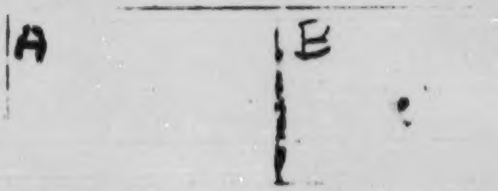
1. Always follow the manufacturer's directions for use of their grouping sera if they differ from the above directions.
2. Artifacts resulting from drying at the periphery of the serum-cell mixture should not be interpreted as indicating specific agglutination.
3. Blood obtained from a capillary puncture may be tested directly on the slide provided that large drops are obtained and mixed quickly with the antisera.
4. Use only fresh cells for testing.
5. Do not heat the slide before or during the test.
6. Do not use applicator sticks more than once.
7. Do not contaminate unlike grouping sera with each other.
8. To help avoid cold agglutination, cells and serum should be at room temperature at time of testing.
9. False negative reactions may be caused by—
   - *a.* Weak grouping sera.
   - *b.* Hemolysis from chemicals in solutions or glassware.
   - *c.* Deteriorated or contaminated serum.
   - *d.* Excessively heavy cell suspensions which absorb all agglutinins, thus preventing visible agglutination.
   - *e.* Weak agglutinogens encountered in the erythrocytes of the newborn and in the subgroups.



STEP 1. Clean properly labeled glass slide.



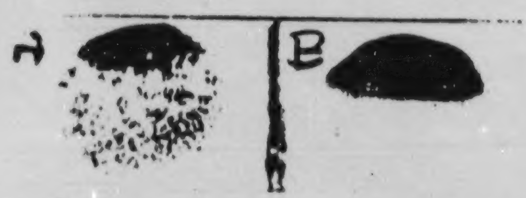
STEP 2. Add one drop of anti-A grouping serum to the side marked "A".



STEP 3. Add one drop of anti-B grouping serum to the side marked "B".

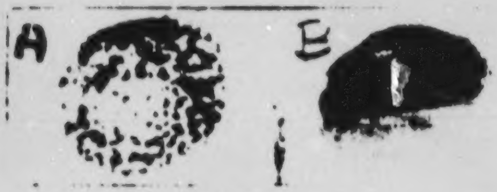
STEP 4. Transfer the cells adhering to the applicator stick to the side marked "A", and mix.

STEP 5. With a clean applicator stick, transfer cells to the side marked "B".

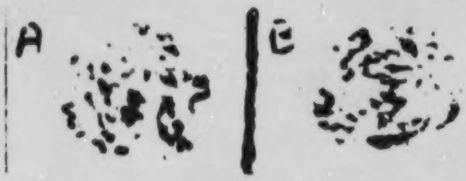


STEP 6. Tilt and rotate the slide. Observe for clumping within 3 minutes.

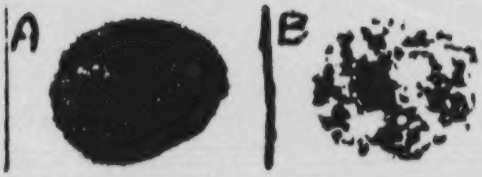
Figure 4-8①. Slide method of blood cell grouping.



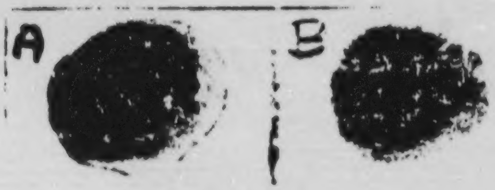
Group A



Group AB



Group B



Group O

Figure 4-8①—continued. Interpretation of slide method of blood cell grouping.

10. False positive reactions may be caused by—

   a. Rouleaux formation.
   b. Cold hemagglutinins in high titer.
   c. Bacteriogenic panagglutination.
   d. Clotting.
   e. Drying.

11. An auto-agglutinin control should be included. It consists of patient's serum used in place of grouping serum. The

remainder of the procedure is identical to the regular test.

12. Known A_1 and B cells should be included as controls to indicate the effectiveness and specificity of the grouping sera. They should be processed in a manner similar to the test specimen.

Test Tube Method

Test tube methods are considered to be more sensitive than slide methods and are less likely to promote rouleaux formation.

APPARATUS:

Test tubes, Kahn.

Medicine droppers.

REAGENTS:

Anti-A grouping serum.

Anti-B grouping serum.

Note. These sera must meet the standards established by the National Institutes of Health (Division of Biologic Standards) for potency, avidity, and specificity.

Normal saline (0.85 percent).

TECHNIQUE:

1. Select two absolutely clean test tubes and mark one "A" and the second "B." Write patient's name or code number on each tube.
2. Place one drop of anti-A grouping serum in tube A and one drop of anti-B grouping serum in tube B.
3. Add one drop of saline suspended patient's cells to each tube. The patient's cells are in 2 percent suspension in saline and are prepared according to instructions given in appendix I.
4. Shake the tubes well and centrifuge them for 1–2 minutes at 1,000-2,000 r.p.m.
5. Gently dislodge the button of cells from the bottom of the tubes and read for agglutination (clumping). In some instances, doubtful results may have to be checked by viewing the test tube through a dissecting microscope or by placing a drop of the mixture on a slide and viewing it with the low power objective of a standard microscope. This technique is summarized in table 4–7.

INTERPRETATION:

The blood group is determined by the presence or absence of agglutination (clumping) with anti-A and anti-B sera as indicated in table 4–8. This procedure is illustrated in figure 4–4 ①, 4–4 ②, and 4–4 ③.

Table 4–7. Test Tube Method of Blood Cell Grouping

Clean Test Tubes Properly Labeled

	A	B
Anti-A Grouping Serum	One Drop	
Anti-B Grouping Serum		One Drop
Blood Cells, 2 Percent Suspension	One Drop	One Drop

Shake tubes and centrifuge for 1-2 minutes at 1000-2000 rpm.
Gently dislodge button of cells and observe for clumping.

REACTION		
Group O	No Clumping	No Clumping
Group A	Clumping	No Clumping
Group B	No Clumping	Clumping
Group AB	Clumping	Clumping

Table 4-8. Interpretation of Blood Grouping Reactions

Blood cells from group	Reaction in anti-A serum	Reaction in anti-B serum
O	No clumping	No clumping.
A	Clumping	No clumping.
B	No clumping	Clumping.
AB	Clumping	Clumping.

DISCUSSION:

1. Always follow the manufacturer's directions concerning the use of grouping serum.
2. The most common error in blood grouping is incorrect labeling of test tubes. The technician should exercise extreme care to avoid this source of error.
3. Always use fresh cell suspensions prepared from recently drawn blood. The cell suspension should only be used within 5 hours after preparation.
4. Do not contaminate unlike grouping sera with one another.
5. To help avoid cold agglutination, cells and serum should be at room temperature at time of testing.
6. False negative reactions may be caused by—
   - *a.* Weak grouping sera.
   - *b.* Hemolysis from chemicals in solutions or glassware.
   - *c.* Deteriorated or contaminated serum.
   - *d.* Excessively heavy cell suspensions which absorb all agglutinins, thus preventing visible agglutination.
   - *e.* Weak agglutinogens encountered in the erythrocytes of the newborn and in the subgroups.
7. False positive reactions may be caused by—
   - *a.* Rouleaux formation.
   - *b.* Cold hemagglutinins in high titer.
   - *c.* Bacteriogenic panagglutination.
   - *d.* Clotting
   - *e.* Drying.
   - *f.* Reading microscopically after prolonged centrifugation.
8. An auto-agglutinin control should be included. It consists of patient's serum used in place of grouping serum. The remainder of the procedure is identical to the regular test.
9. Known A_1 and B cells should be included as controls to indicate the effectiveness and specificity of the grouping sera. They should be tested in a manner identical to that applied to the test specimens.

SERUM GROUPING

The proper group of a blood specimen is ordinarily determined by testing the red cells with known anti-A and anti-B sera. In order to assure accurate blood groupings, the cell grouping results are confirmed by testing the patient's serum with known group A_1 and B cells. From the basic makeup of the ABO blood groups, it is known that when an agglutinogen is present, the corresponding agglutinin is absent. Conversely, when an agglutinogen is absent, the corresponding agglutinin is present. Thus, tests, using the individual's serum and known cells, confirm tests based on the reaction of the person's cells with known antiserum.

APPARATUS:

Test tubes, Kahn.

Medicine droppers.

REAGENTS:

Known A_1 cells (pooled cells from three or more group A individuals).

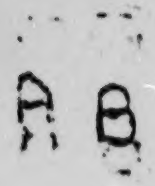
Known B cells.

Note. The above cells are prepared in a 2 percent suspension in saline. Instructions for preparing these suspensions are included in appendix I.

Normal saline, 0.85 percent.

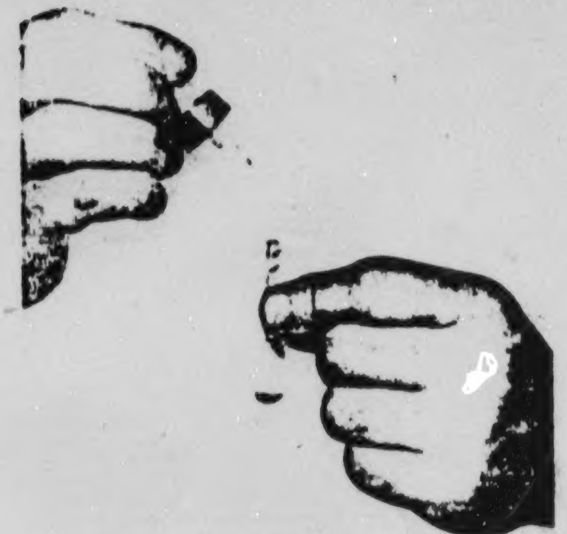
TECHNIQUE:

1. Serum to be tested is heated in a 56° C. water bath for 10 minutes in order to inactivate any complement which might be present. The presence of complement may cause hemolysis of cells and give inaccurate results.
2. Select two absolutely clean test tubes and mark one "A" and the second one "B." Write the patient's name or code number on each tube. The tubes should be clearly designated as being used for serum grouping.

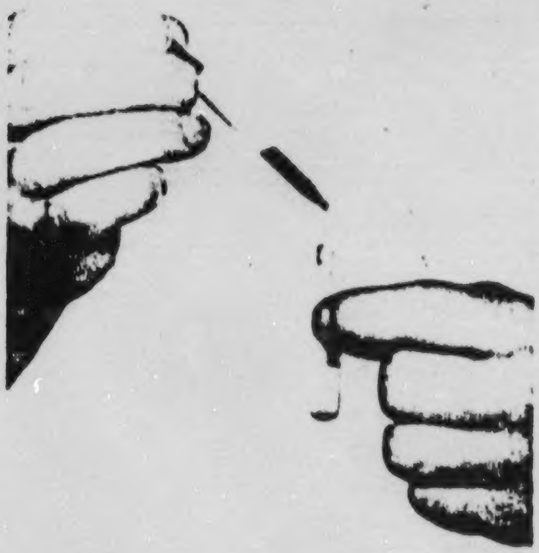


STEP 1. Clean properly labeled test tubes.

STEP 2. Place one drop of anti-A grouping serum in the tube marked "A".



STEP 3. Place one drop of anti-B grouping serum in the tube marked "B".



STEP 4. Add one drop of 2 per-cent suspension of blood cells to the tube marked "A".

Figure 46. Test tube method of blood cell grouping.

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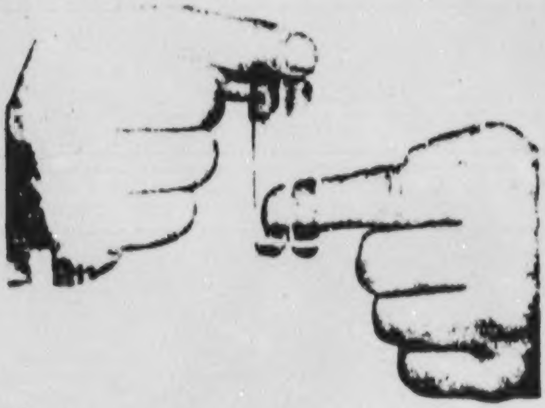
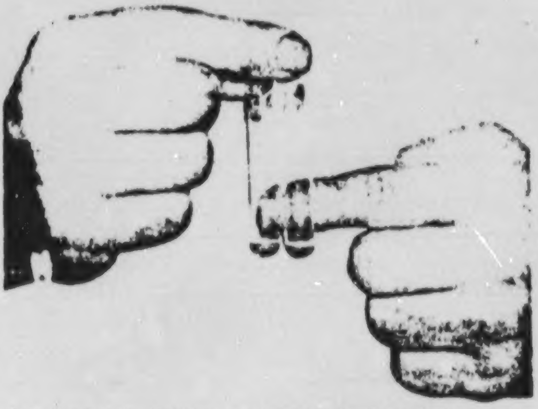
STEP 5. Add one drop of a 2 per-cent suspension of blood cells to the tube marked "B".

STEP 7. Centrifuge the tube to loosely pack the cells.



STEP 6. Gently shake the tubes.



STEP 8. Gently dislodge the buttons of cells and observe for clumping.

*Figure 4-4*③—**continued.**

3. Place two drops of serum to be tested into each tube.
4. Add two drops of a freshly prepared 2 percent saline suspension of known pooled A_1 cells to the tube marked "A."
5. Add two drops of a freshly prepared 2 percent saline suspension of known B cells to the tube marked "B."
6. Shake the tubes well.
7. Centrifuge the tubes for 3 minutes at 2,000 r.p.m.
8. Interpret macroscopically. In reading, avoid vigorous shaking since it may break up agglutination. This technique is summarized in table 4–9.

INTERPRETATION:

The significance of agglutination (clumping) can be interpreted as indicated in table 4–10. This procedure is illustrated in figure 4–5①, 4–5②, and 4–5③.

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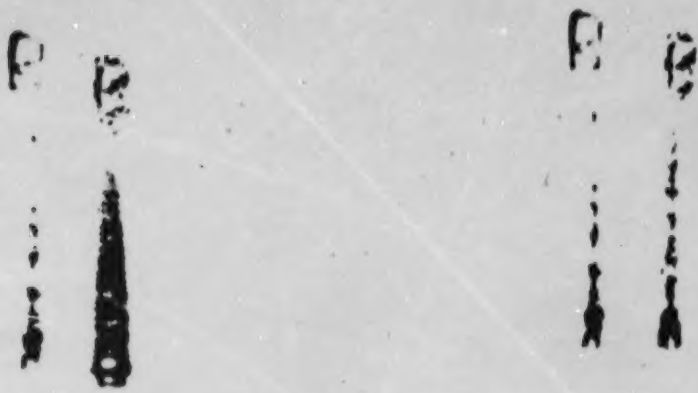
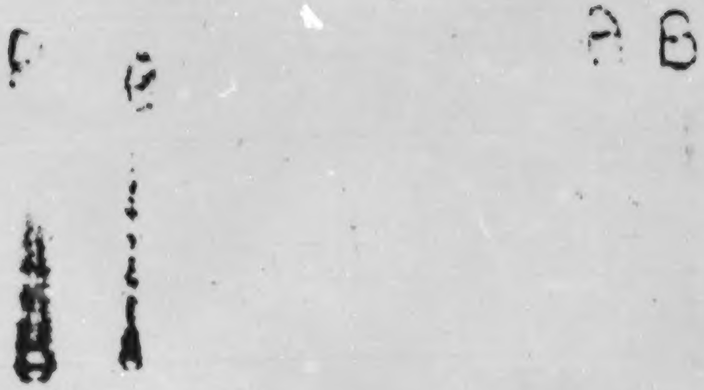


Group A **Group AB**



Group B **Group O**

Figure 4-4③—continued. Interpretation of test tube method of blood cell grouping.

DISCUSSION:

1. Test cells must be fresh and properly prepared.
2. For best results, the cells should be washed free of their serum.
3. The A cells used should be a pool of group A or single group A_1 cells.
4. Make sure that all tubes are properly labeled.
5. Difficulty in confirming the blood group will frequently be encountered in the case of infants whose agglutinins are not well developed at time of birth and in the early months of postnatal life. The cell grouping procedures, run in duplicate using different lots of grouping sera, will suffice to confirm the infant's blood group.
6. To help avoid cold agglutination, cells and serum should be at room temperature at time of testing.
7. False negative reactions may be caused by—
    - *a.* Hemolysin or complement in serum under test.
    - *b.* Hemolysis from chemicals in solutions or glassware.
    - *c.* Deteriorated or contaminated test cells.
    - *d.* Deteriorated or contaminated serum specimen.
    - *e.* Excessively heavy cell suspensions which absorb all agglutinins, thus preventing visible agglutination.

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Table 4-9. Serum Grouping of Blood

Clean Test Tubes Properly Labeled

Serum Under Test (inactivated)	Three Drops	Three Drops
Group A Cells 2 Percent Suspension	Three Drops	
Group B Cells 2 Percent Suspension		Three Drops

Shake the tubes well. Centrifuge for 3 minutes at 1500 rpm. Gently dislodge button of cells. Observe macroscopically for clumping.

REACTION		
Group A	No Clumping	Clumping
Group B	Clumping	No Clumping
Group AB	No Clumping	No Clumping
Group O	Clumping	Clumping

NOTE: Known Anti-A and Anti-B serums should be tested simultaneously as controls.

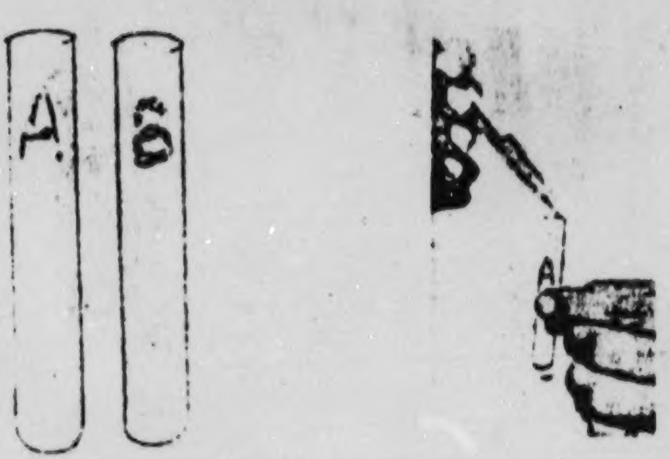
Table 4-10. Interpretation of Serum Grouping Reactions

Blood group	Antibody present in serum	Reaction with A cells	Reaction with B cells
A	Anti-B	No clumping	Clumping.
B	Anti-A	Clumping	No clumping.
AB	None	No clumping	No clumping.
O	Anti-A and anti-B.	Clumping	Clumping.

8. False positive reactions may be caused by—
   - *a.* Rouleaux formation.
   - *b.* Cold agglutinins in high titer.
   - *c.* Bacteriogenic panagglutination.
   - *d.* Clotting.
   - *e.* Drying.
   - *f.* Reading microscopically after prolonged centrifugation.
9. An auto-agglutinin control should be included. It consists of patient's cells used in place of the known A or B cells. The procedure is identical to the regular test.
10. Known anti-A and anti-B grouping sera should be included as controls to indicate the specific reactivity of the test cells. These sera should be processed in a manner similar to that applied to the serum under test.

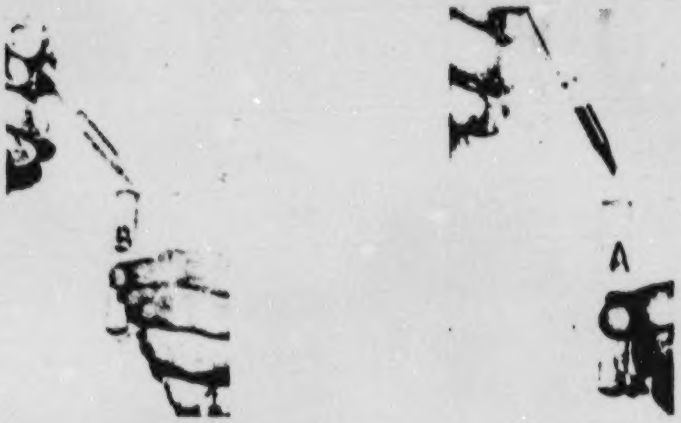
ABO SUBGROUPS

In 1911 it was discovered that the four basic blood groups were more complex than originally thought. Groups A and AB, it was found, could be divided into subgroups. The most frequently occurring subgroups of A are A_1 and A_2. The subgroups of AB which occur most commonly are A_1B and A_2B. These divisions increase the number of common groups from four to six: O, A_1, B, A_1B, A_2, A_2B. Other more weakly reacting subgroups have been described but these occur quite rarely. The rare subgroups include A_3, A_4, A_5, A_6, A_m, A_0, A_x, and A_z. In addition to the common subgroup agglutinogens A_1 and A_2, subdivisions of the anti-A agglutinin have also been discovered. These include anti-A proper which will agglutinate all subgroups of A, and anti-A_1 which agglutinates only cells from subgroup A_1.



STEP 1. Clean properly labeled test tubes.

STEP 2. Place three drops of patient's inactivated serum in the tube marked "A".



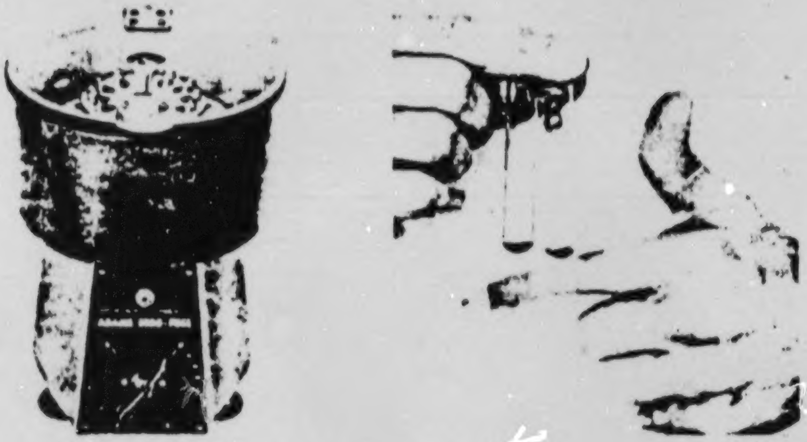
STEP 3. Place three drops of patient's inactivated serum in the tube marked "B".

STEP 4. Add three drops of a 2 percent suspension of group "A" cells to the tube marked "A".

Figure 4-6①. Serum grouping of blood.

STEP 5. Add three drops of a 2 percent suspension of group "B" cells to the tube marked "B".

STEP 6. Shake the tubes well.



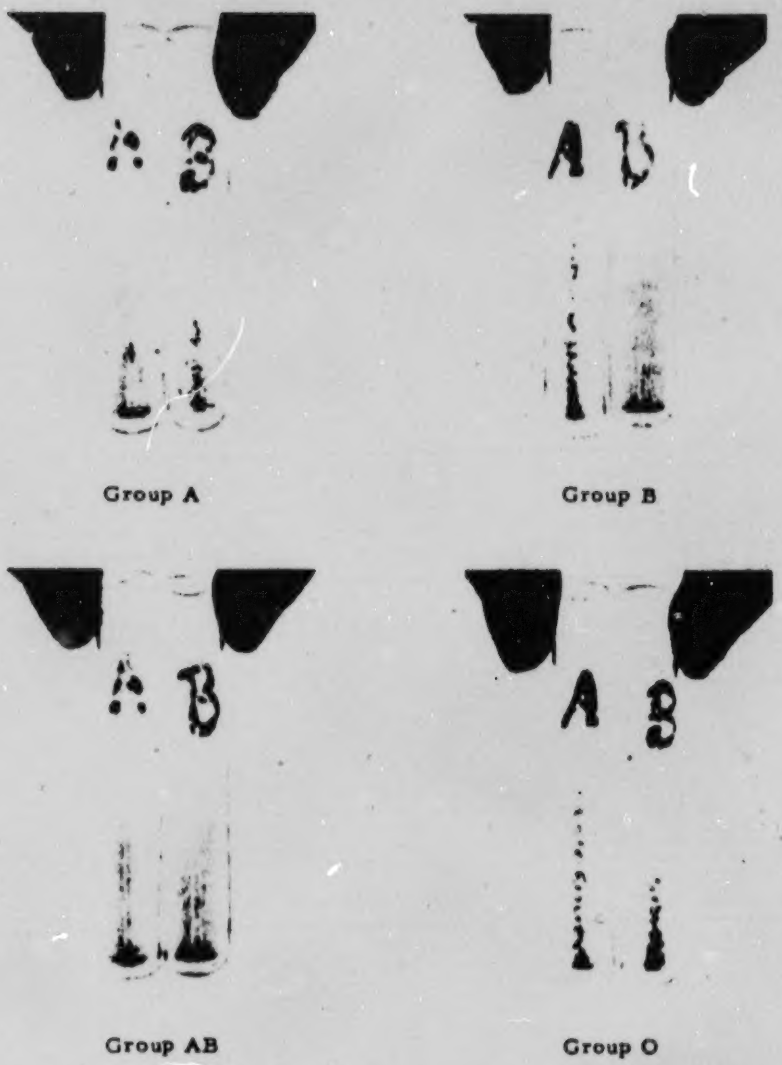
STEP 7. Centrifuge the tube to loosely pack the cells.

STEP 8. Gently dislodge the buttons of cells and observe for clumping.

Figure 4-5③—continued. Serum grouping of blood.

Blood transfusion reactions from subgroup incompatibility have been reported, but isosensitization from such transfusions is rather rare. Fortunately, these reactions have been of a mild nature due to the low titer of the antibodies and to their thermal requirements which are below body temperature. The real danger in misidentification of subgroups is in mistaking weakly reacting subgroups A_2, A_3, A_4, A_5, etc., for group O. Such blood might be employed as a so-called universal donor with a resulting severe hemolytic reaction. Even should it not be so used, the excessive amount of time lost in attempting to crossmatch such blood might well, in some cases, make the difference between life and death.

In order to differentiate between A_1 and the other subgroups of blood group A (A_2, A_3, etc.,),



Group A

Group B

Group AB

Group O

Figure 4-5③—continued. Interpretation of serum grouping of blood.

a special serum is required. Such a serum is called absorbed anti-A serum. It is prepared from serum of a group B donor from which the anti-A proper agglutinin has been removed. This leaves only the anti-A_1 agglutinin which agglutinates only A_1 cells. This serum is available from commercial sources or it can be prepared in the laboratory. Instructions for its preparation are included in appendix 1.

Either a slide test or tube test can be employed to determine subgroups of A and AB. In some doubtful cases, it is recommended that both be performed. An additional technique for determining blood subgroups utilizes extracts from the seeds of certain plants. The slide and tube serum procedures, along with the plant hemagglutinin technique, will be presented below. A general discussion of precautions and sources of error in subgrouping procedures will be found following the presentation of the techniques.

Slide Test for Determination of Subgroups

The slide test for determination of subgroups is a simple, convenient procedure which can be performed in a brief period of time. It can be run by itself or used in conjunction with the test tube technique.

APPARATUS:

Boerner slides (2¼ x 4¼ in. slides with 10 mold-pressed cells).

Medicine droppers.

Applicator sticks.

REAGENTS:

Normal saline, 0.85 percent.

Absorbed anti-A serum. (Commercially prepared antiserum should be used if at all possible.)

Known A_1 and A_2 cells, 10 percent suspension. (Instructions for preparing these suspensions are included in app I.)

TECHNIQUE:

1. Prepare a fresh 10 percent suspension of patient's cells in saline. (Instructions for preparation of this cell suspension are included in app I.)
2. Place one drop of absorbed anti-A serum in the well of a Boerner slide.
3. Add one drop of the 10 percent cell suspension. Mix with a clean wooden applicator stick.
4. Rotate and tilt the slide and read for agglutination.
5. The known A_1 and A_2 cells prepared as 10 percent suspensions in saline should be tested simultaneously as controls. This technique is summarized in table 4-11.

INTERPRETATION:

1. Group A_1 cells begin to exhibit agglutination after 1 minute. Gross agglutination will be visible within a 3 minute period. A_1B cells may react somewhat more slowly.
2. Cells from group A_2 blood will show no reaction for 3 minutes but will develop a granular appearance after prolonged standing.
3. The tests should not be read after 3 minutes.
4. Cells of weakly reacting subgroups may be missed in the slide test. In order to differentiate these, the tube technique with centrifugation is the recommended procedure.

Tube Test for Determination of Subgroups

The tube test for the determination of subgroups is considered to be more sensitive than the slide test. It may be employed routinely or used to confirm results obtained in the slide procedure.

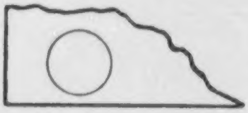
APPARATUS:

Test tube, Kahn.

Medicine droppers.

Table 4-11. Slide Test for Determination of Subgroups

Well of Clean Boerner Slide



Absorbed Anti-A Grouping Serum	One Drop
Blood Cells, 10 Percent Suspension	One Drop

Mix with applicator stick. Rotate and tilt slide. Observe for clumping.

INTERPRETATION	
Group A_1, A_1B	Clumping Within 3 Minutes
Group A_2, A_2B	No Clumping Within 3 Minutes

NOTE: Known Group A_1 and A_2 cells should be tested simultaneously as controls.

REAGENTS:

Normal saline, 0.85 percent.

Absorbed anti-A serum.

Known A_1 and A_2 cells, 2 percent suspension. (Instructions for preparing these suspensions are included in app I.)

TECHNIQUE:

1. Prepare a fresh 2 percent suspension of patient's cells in saline. (Instructions for preparing this suspension are included in app I.)
2. Select an absolutely clean test tube and into it place one drop of absorbed anti-A serum.
3. Add two drops of the 2 percent suspension of patient's red blood cells.
4. Mix the contents of the tube thoroughly and hold the serum-cell mixture at room temperature for no longer than 15 minutes.
5. During the 15-minute incubation period, observe for macroscopic clumping.
6. The known A_1 and A_2 cells prepared as 2 percent suspensions in saline should be tested simultaneously as controls. This technique is summarized in table 4-12.

INTERPRETATION:

1. Agglutination will be present with specimens of the A_1 or A_1B subgroups. The red blood cells of subgroups A_2 and A_2B will exhibit a negative reaction after 15 minutes.
2. Light centrifugation (1,000 r.p.m. for 1 minute) may be used to make reading easier. Following centrifugation, results of the test are read immediately after swirling the contents of the tube to dislodge the compacted cells. Observe macroscopically for agglutination which will persist with blood cells possessing the A_1 agglutinogen. Erythrocytes of subgroups A_2 and A_2B will resuspend evenly with no visible clumping.
3. Blood cells of weakly reacting subgroups may exhibit gross agglutination only after centrifugation.

Use of Plant Hemagglutinins for Determining Subgroups

The seeds of certain plants can be used to differentiate group A_1 from group A_2 bloods. Lima beans and seeds of another legume, Dolichos biflorus, when properly prepared, grossly agglutinate A_1 and A_1B blood cells. A_2 and A_2B cells are either not agglutinated or give a very weak reaction.

APPARATUS:

Test tubes, Kahn.

Medicine droppers.

Applicator sticks.

Table 4-12. Tube Test for Determination of Subgroups

Clean Test Tube Properly Labeled

Absorbed Anti-A Grouping Serum	One Drop
Blood Cells, 2 Percent Suspension	Two Drops

Mix the contents of the tube. Incubate at room temperature for 15 minutes. Observe for macroscopic clumping.

INTERPRETATION	
Group A_1, A_1B	Clumping after 15 minutes
Group A_2, A_2B	No clumping after 15 minutes

NOTE: Known A_1 and A_2 cells should be tested simultaneously as controls.

REAGENTS:

Plant seed extract prepared from lima beans or seeds of Dolichos biflorus. (Complete instructions for preparing and titrating this reagent are included in app I.)

Known A_1 and A_2 cells.

TECHNIQUE:

1. Select a clean test tube and place into it two drops of prepared plant seed extract.
2. Add cells from clotted blood or whole blood in anticoagulant to the plant seed extract by dipping a wooden applicator stick into the blood and transferring the adhering cells to the test tube.
3. Place the tube in a 37° C. water bath for 10 minutes.
4. Centrifuge the test tube at 1,000 r.p.m. for 2 minutes.
5. Following centrifugation results of the test are read immediately after swirling the contents of the tube to dislodge the compacted cells.
6. Known A_1 and A_2 cells should be tested simultaneously as controls. This technique is summarized in table 4-13.

INTERPRETATION:

Blood cells from group A_1 and A_1B will exhibit gross agglutination. A_2 and A_2B bloods will give a negative or very weak reaction.

DISCUSSION:

1. It is essential to interpret results at the exact termination of the timing period.
2. If the absorbed anti-A serum or plant seed extract is prepared in the laboratory, great care must be taken to follow the instructions closely. (Commercially prepared reagents should be used if at all possible.)
3. Avoid incorrect labeling of test tubes or slides.
4. Use fresh cell suspensions prepared from recently drawn blood.
5. Always follow the manufacturers' instructions for using their grouping sera.
6. To avoid cold agglutination, cells and serum should be at room temperature at time of testing.
7. False negative reactions may be caused by—
   - *a.* Weak grouping sera.
   - *b.* Hemolysis from chemicals in solutions or glassware.
   - *c.* Deteriorated or contaminated serum.
   - *d.* Excessively heavy cell suspensions which absorb all agglutinins, thus preventing visible agglutination.
   - *e.* Week agglutinogens encountered in the erythrocytes of the newborn.

Table 4-13. Plant Hemagglutinins for Determining Subgroups

Clean, Properly Labeled Test Tube

Prepared Plant Seed Extract	Two Drops
Blood Cells (Whole Blood)	Quantity adhering to tip of applicator stick

Mix. Incubate at 37°C. for 10 minutes. Centrifuge at 1000 rpm for 2 minutes. Observe for clumping.

INTERPRETATION	
Group A_1, A_1B	Clumping
Group A_2, A_2B	No clumping or very weak reaction

NOTE. Known A_1 and A_2 cells should be tested simultaneously as controls.

8. False positive reactions may be caused by—
   - *a.* Rouleaux formation.
   - *b.* Cold hemagglutinins in high titer.
   - *c.* Bacteriogenic panagglutination.
   - *d.* Clotting.
   - *e.* Drying.
   - *f.* Reading results microscopically after prolonged centrifugation.

9. An auto-agglutinin control should be included. It consists of patient's serum in place of grouping serum or plant seed extract. The remainder of the procedure is identical to the regular test.

10. The known A_1 and A_2 cells tested in the subgrouping procedures serve to indicate the effectiveness and specificity of the absorbed anti-A serum or plant seed extract employed.

DETECTION OF WEAKLY REACTING SUBGROUPS

Occasionally, the blood bank technician will encounter blood specimens containing the very weakly reacting A subgroup antigens, (A_4, A_5 A_0, etc.). It has been demonstrated that the strongest anti-A sera available will not give a strong reaction with red cells possessing such factors. Sera drawn from individuals who are group O (i.e., anti-A, B serum) will give stronger reactions with these A_4, A_5, or A_0 cells than will anti-A sera of much higher titer. This situation suggests that A cell contains another antigen of the series and that the group O individual has the corresponding antibody for this antigen. In any event, the use of group O (anti-A, B) serum will be helpful in instances where a weak A subgroup is suspected. The anti-A, B serum may detect this subgroup antigen which has not been detected in tests with anti-A serum.

CRITERIA FOR BLOOD GROUPING SERA

When performing blood grouping tests, it is essential that the highest quality grouping sera be used. These sera should be prepared in laboratories licensed by the National Institutes of Health (Division of Biologic Standards) and meet the requirements established by that agency. Satisfactory grouping serum must pass tests for specificity, avidity, and specific agglutinin titer.

Specificity means that a serum has been proved free of hemolysins, irregular agglutinins, and iso-hemagglutinins other than those specifically mentioned on the bottle label. Anti-A blood grouping serum should agglutinate cells of group A, including subgroups A_1. and A_2, and AB, including subgroups A_1B and A_2B. Anti-A serum should not, however, cause agglutination of group O cells or group B cells. Anti-B grouping serum must agglutinate cells of groups B and AB, including subgroups A_1B and A_2B. Anti-B serum should not agglutinate cells of group O or of group A. Grouping sera should be free of excessive tendency to produce rouleaux formation.

Avidity refers to the ability of a blood grouping serum to react promptly and intensely. When anti-A grouping serum is mixed on a slide with an equal volume of 10 percent A cell suspension in saline, visible agglutination should appear within 30 seconds using A_2 cells and within 45 seconds using A_2B cells. Anti-B grouping serum should cause visible agglutination within 15 seconds when mixed with B cells. In all the tests, the cell clumps observed in the test mixture should be 1 square mm. or larger 3 minutes after mixing.

Specific agglutinin titer refers to a grouping serum which is rich in active specific iso-hemagglutinin. The meaning of titer has already been explained. Anti-A blood grouping serum should possess a titer against A_1 cells of 1:256 or more; against A_2 cells, 1:128 or more; and against A_2B cells, 1:64 or more. Anti-B serum should have a titer against B cells of 1:256 or more. The above titers are very conservative readings. In practice, titers of good blood grouping sera will be much higher.

SOURCES OF ERROR IN BLOOD GROUPING

Improper Use of Reagents

Strict adherence to manufacturer's instructions is essential in performing serological tests.

Natural Variations in Cells

Ordinarily strong, clearcut reactions are observed between blood specimens and anti-A and anti-B grouping sera. Exceptions do occur and the technician should be aware of them. Occasionally, weak reactions will be observed with cells of group A (subgroup A_2) and with cells of group AB (subgroup A_2B or A_3B). Such a reaction might be overlooked with the false designation of cells of group A as O or cells of group AB as B. This error can be avoided in several ways. They include—

1. Carefully observing and noting weak reactions.
2. Using 2 percent suspensions of cells in saline. This medium favors the detection of weak reactions by promoting agglutination and facilitating microscopic examination.
3. Employing the test tube technique which shows stronger reactions than the slide method.
4. Employing serum grouping.
5. Employing anti-A,B (group O) serum.

Bacterial Contamination of Serum

Contamination of serum by bacteria may cause the appearance of bacteriogenic panagglutinins which will agglutinate all cells. This contamination may also weaken the serum dangerously by destroying the desired iso-hemagglutinins. Clean, but not necessarily aseptic technique, should be used when opening the serum containers. Care should be taken to protect the dropper cap from contamination and to return it promptly to the bottle. The dropper provided is the only object which should be introduced into the container. The grouping serum should be stored at 2°-10° C. at all times when it is not in use. This will retard growth of any bacteria which gain access to the serum.

Note. Auto-agglutination and pseudoagglutination have been discussed in detail in chapter 2.

Chemical and Biochemical Deterioration

Avoid contact of the serum with traces of chemicals such as might be present in improperly cleaned pipettes, slides, and test tubes. It is of utmost importance to avoid any mixing of the blood grouping sera with each other.

Unsatisfactory Blood Specimens

Changes induced by bacterial contamination may cause cells to clump in all sera. In addition, spoiled specimens will exhibit gross hemolysis. A freshly drawn specimen, if not stored in the refrigerator, may undergo deterioration within a few hours. Freezing of the cells will hemolyze the blood and must also be avoided. If a blood specimen is obtained from an individual who has recently been infused with blood of another group, the technician can expect to encounter a mixture of patient's and donor's erythrocytes. In a mixture of this sort, each cell type will exhibit its own group reactions. The predominant cells will usually overlay the others and the true nature of the reaction will only be visible by reading the reaction under the microscope.

Cell Suspensions

Red blood cells suspended in saline may undergo rapid deterioration. It is essential then, that the cell suspensions be used as soon as possible after preparation and certainly no later than the day of preparation.

Hemolysis

If unsuitable blood grouping serum is employed, hemolysis rather than agglutination of the blood cells may occur. The lysis may be partial or complete and can be identified in the test tube technique by red coloration of the supernatant fluid after centrifugation. In the slide test, hemolysis is identified by a noticeable reduction in the number of cells on the slide. This will be accompanied by a transparent red film on the slide which will be observed when the slide is tilted.

Faulty Identification

Error in identification of a blood specimen is a constant possibility. All tubes of patient's blood to be employed in blood grouping procedures must be carefully labeled. A gummed label or piece of adhesive tape with the patient's full name and hospital identification should be used. Do not use wax pencils for identifying patient's blood specimen tubes.

CHAPTER 5

RH TYPING PROCEDURES

HISTORICAL SYNOPSIS

For a long time after Landsteiner's description of the four basic blood groups, it was suspected that additional specific differences in blood might be responsible for many of the transfusion reactions which were still being observed. In 1939, supporting evidence for this view was obtained. Levine and Stetson suggested an explanation for the origin of an atypical agglutinating antibody considered to be the cause of a severe reaction in a woman patient. The woman, who had recently delivered, experienced the reaction at the time of her very first transfusion. The serum of this group O patient agglutinated the cells of about 80 percent of other group O individuals. The suggestion was made that the fetus may have inherited from the father a dominant agglutinable factor, which was not present in the blood of the mother. As a result, immunization (also called sensitization) of the mother could have occurred from the transplacental passage of minute amounts of fetal erythrocytes into the maternal circulation. At this point, no name had been given to the specific antigen involved.

In 1940 Landsteiner and Wiener reported the results of experiments involving blood of the monkey, Macaca rhesus. In the experiments, rabbits were injected with erythrocytes from the monkey. An antiserum prepared from the rhesus-immunized rabbits agglutinated not only the monkey's cells but also the erythrocytes of approximately 85 percent of individuals of the Caucasian race. The specific factor present on the monkey blood cells, as well as the blood cells of most human beings, was termed the "Rh" or Rhesus factor. This factor was later found to be identical to the one originally described by Levine and Stetson. Those bloods possessing the factor were termed Rh positive while those lacking the antigen were designated Rh negative.

It soon became apparent that immunization or sensitization could occur in several ways. These include transfusion of Rh negative recipients with Rh positive blood, an Rh negative mother carrying an Rh positive fetus, and the intramuscular injection of whole blood. It was emphasized earlier that the ABO blood system is characterized by naturally occurring antibodies. This is not the case in the Rh system and the Rh antibodies appear only after immunization or sensitization has taken place.

Although it was originally thought that the Rh factor was a single antigen, it is now known to be, in reality, a mosaic of several antigens. As a result, what was once considered to be a simple blood factor has developed into a subject of ever-increasing complexity. For satisfactory performance in the laboratory, the blood bank technician does not require a complete and detailed understanding of the Rh system. The basic information presented here should be sufficient to provide an adequate working knowledge.

CLASSIFICATION AND NOMENCLATURE

A basic group of six closely related antigens makes up the Rh system. Fisher and Race have designated these as D and d, C and c, and E and e. Wiener has employed the symbols Rh_o and Hr_o, rh′ and hr′, and rh″ and hr″ to designate the same antigens. Each factor, except d (Hr_o), has been discovered by means of a reaction with its specific antibody, produced in individuals immunized through pregnancy or transfusion. The antibody whose formation is stimulated most frequently is anti-D (anti-Rh_o) which reacts with the erythrocytes from 85 percent of the white U.S. population, in so doing, reveals D (Rh$_o$), the original Rh antigen. The antigen d (Hr$_o$) is a theoretical factor not yet established.

The following basic anti sera, anti-C, anti-D, anti-E, anti-c, anti-d, anti-e, are commercially available. The two principal systems of nomenclature for the Rh antibodies and the frequency with which they react in a random Caucasian population are indicated in table 5-1.

D or Rh$_o$, the original Rh factor, is the most antigenic. The terms "Rh positive" and "Rh negative," in a clinical sense, refer solely to the presence or absence of D (Rh$_o$). Individuals who possess C (rh') or E (rh") but are only tested for and found to lack D (Rh$_o$) are always listed as Rh negative. For blood donation purposes, however, an individual should be considered Rh negative only when he is negative for all three factors.

Table 5-1. Basic Anti-Rh Sera and Frequency of Reaction

Nomenclature		Percent positive reactions	Percent negative reactions
Fisher-Race	Wiener		
Anti-D	Anti-Rh$_o$	85	15
Anti-d	Anti-Hr$_o$	63	37
Anti-C	Anti-rh'	70	30
Anti-c	Anti-hr'	80	20
Anti-E	Anti-rh"	30	70
Anti-e	Anti-hr"	97	3

INHERITANCE OF Rh ANTIGENS

The gene responsible for the development of the antigen D is also referred to as gene D. Every individual inherits the gene D or its alternate d from each of his parents. This is also true as regards C and c and E and e. For example, if D is not present, then d will be present; if e is absent, E is present; or if C is absent, c is present. Thus, when one character is absent, its alternate or allelomorphic form will be present, rather than there being a vacant locus on the chromosome. Recently, a fourth and fifth set of allelomorphs have been reported. They are F and f and G and g. These additional factors have not, as yet, assumed a role of practical importance in routine blood banking.

In man there are 23 different chromosomes. Each individual has a pair of each of these different chromosomes. One chromosome of each pair is derived from the mother and the other is contributed by the father. The individual will, therefore, have two chromosomes carrying genes for the Rh system.

There are eight possible ways in which C or c, D or d, and E or e can be arranged in a single chromosome. Listed according to their frequency of occurrence, starting with the most common, they are—

1. CDe
2. cde
3. cDE
4. CDE
5. cDe
6. cdE
7. Cde
8. CdE

In the somatic cells there are two such chromosomes and the most common genotype combinations are indicated in table 5-2.

Table 5-2. Common Rh Genotypes
(From a study of 2,000 English bloods)
(Race, R. R., Mourant, A. E., Lawler, S. D., and Sanger, R.)

Rh positive		Rh negative	
Genotype	Incidence percent	Genotype	Incidence percent
CDe/cde	32.7	cde/cde	15.1
CDe/CDe	17.7		
CDe/cDE	11.9	cdE/cde	0.9
cDE/cde	11.0		
CDe/cDe	2.1	Cde/cde	0.8
cDE/cDE	2.0		
cDe/cde	2.0		
CDe/cdE	1.0		
CDe/Cde	0.8		

Other combinations occurring much less commonly contribute to a total of 36 possible different genotypes.

Rare bloods exist which fail to exhibit any reaction with anti-C, anti-c, anti-E, or anti-e antiserums. The condition has been termed a deletion of genes. So far these exceptions have been of the types indicated below.

1. The individual types as D (Rh_o) positive. The erythrocytes, however, are not agglutinated by anti-C (rh′), anti-c (hr′), anti-E (rh″), or anti-e (hr″) sera. The D (Rh_o) antigen is very strongly reactive. It even reacts with high protein active, incomplete antibodies in the absence of high protein media. This is called Rh_o or (__D__).
2. The individual types as D (Rh_o) positive. The cells react with anti-C (Rh′) and anti-c (hr′) but not with anti-E (rh″) nor anti-e (hr″). This blood is referred to as Rh_1^w (CD__).
3. The red cells of an individual fail to react with anti-D, anti-C, anti-E, anti-c, anti-e, anti-f, anti-C^w, anti-C^x, anti-Ce (rh_i), anti-hr^s and anti-G. The phenotype can be represented as ___/___ or as suggested by Ceppellini as "Rh_{null}."

Rh genotype studies have practical application in several ways. These include—

1. Determining whether an Rh positive father is homozygous or heterozygous. (If the individual is homozygous, and erythroblastosis fetalis has already occurred in a severe form, there is little hope for unaffected future pregnancies.) This situation will be discussed in detail in chapter 6.
2. Anthropological and family studies (in conjunction with ABO, Kell and other systems) involving—
   - *a.* Monozygosity or dizygosity of like-sexed twins.
   - *b.* Paternity suits.
   - *c.* Interchange of infants in a nursery.
   - *d.* Other medicolegal identification studies.

VARIETIES OF Rh ANTIBODIES

Rh antibodies may be divided into three distinct categories. In the present discussion these categories will be referred to as first order, second order, and third order antibodies.

First order antibodies are also referred to as saline agglutinins, bivalent antibodies, or complete antibodies because of their reactive properties. These antibodies react most strongly with specific antigen in a saline medium. First order antibodies react less strongly in a protein medium. They are referred to as complete antibodies because they are considered to possess two receptor sites, one at each end of the antibody molecule.

Second order antibodies are sometimes called albumin antibodies, incomplete antibodies, or univalent antibodies. These antibodies react visibly with specific antigen in a protein medium or antiglobulin serum (antiglobulin serum will be discussed in ch 7). In saline they merely combine with the antigen but do not produce a visible reaction. Second order antibodies are incomplete in the sense that they are considered to possess only one receptor site. The remaining ends of the antibody molecules must be "bridged" together by a nonspecific reaction involving protein colloid or antiglobulin serum.

Third order antibodies are also known as atypical antibodies or antiglobulin active antibodies. Third order antibodies react visibly with specific antigen in an antiglobulin medium only. In saline or simple protein, they merely combine with the antigen but do not produce a visible reaction. Third order antibodies are incomplete in the sense that they are believed to possess only one receptor site. The remaining ends of the antibody molecules must be bridged together by a nonspecific reaction involving antiglobulin.

Commercial Rh antiserum is available with either saline active or high proten active agglutinins. The correct procedure and medium must be employed for whichever antibody is specified on the container label. Rh typing procedures utilizing the various orders of antibodies are presented in the following procedures.

Rh TYPING SLIDE (MODIFIED TUBE) ANTISERA

Rh typing employing albumin active antibodies can be used to identify D (Rh_o), C (rh′), e (rh″), or other Rh factors present on erythrocytes. With some antisera a slide procedure can be performed routinely with a tube test performed only in doubtful cases. With other antisera it will be found desirable to test routinely all blood specimens using the tube technique. To determine the best method of testing to use, the antiserum manufacturer's instructions should always be consulted.

Slide (Modified Tube) Method

The slide method employs slide test serum. This procedure may be performed routinely on all bloods and confirmed with the test tube technique in case of doubtful results.

APPARATUS:

Microscope slides (25 mm. x 75 mm.).
Medicine droppers.
Applicator sticks, wood.
Heated Rh viewbox.

REAGENTS:

Anti-Rh typing serum (anti-D (Rh_o) or other depending upon the antigen of interest).
Normal saline, 0.85 percent.

TECHNIQUE:

1. Carefully label a clean, scratch-free microscope slide with the patient's name or code number.
2. Place the slide on the viewbox in order to to warm the slide to a temperature of at least 37° C. (but not over 45° C.). The box surface temperature should be between 45°-50° C.
3. Place one drop of anti-Rh slide test serum on the warm slide.
4. With a dropper, place two drops of whole blood on the slide. The drops should be equal in size to the drop of antiserum. Cells must be suspended in concentrations of approximately 40-50 percent in plasma or serum. Saline suspensions must never be used with slide test antiserum.
5. Using an applicator stick, mix the cells and antiserum and spread the mixture over most of the slide.
6. At 10-second intervals, tilt the viewbox and the slide so that most of the liquid accumulates at one end of the slide, then return the slide to the horizontal position. Alternate the direction of tilting each time.
7. Examine the mixture for macroscopic clumping which should begin to appear within 30 seconds and be complete in 2 minutes. This technique is summarized in table 5-3 and the procedure is illustrated in figure 5-1.

INTERPRETATION:

Clumping of the cells indicates that the blood possesses the Rh antigen. Failure to clump indicates that the Rh antigen is absent. This interpretation can be applied to the presence or absence of any Rh antigen depending on which specific slide test antiserum is employed in the test. If results in the slide test are doubtful, verify with the Rh tube test. All presumptively D (Rh_o) negative tests should be incubated for an additional 50 minutes and converted to the Coombs test for the detection of the weakly reacting D^u factor.

DISCUSSION:

1. Rouleaux formation may occur with this method. It can be dispersed by adding one drop of normal saline and mixing with a clean applicator stick.
2. Warming the slide as directed is essential for prompt and clearly interpretable clumping of the cells.
3. If clotted blood is used, cells may be obtained from the clot and suspended in heavy concentration in the serum surrounding the clot. For the slide test, two parts cells should be mixed with one part serum. Blood taken from the finger or ear may be used. Make a clean skin puncture to insure a good flow of blood without squeezing. Take the blood directly onto the slide and mix at once with anti-Rh typing serum before clotting commences.
4. Excessive concentrations of potassium oxalate interfere with agglutination of red cells by specific antiserum. One drop of a 2 percent solution of ammonium-potassium oxalate is adequate for the collection of 1 ml. of blood. A 20 percent solution should never be used.

Table 5-3. Slide Method-Albumin Active Rh Antibodies
Table III. Slide Method-Albumin Active Rh Antibodies.

Clean, Scratch-free, Properly Labeled Microscope Slide (Warmed on viewbox to at least 37°C. but not over 45°C.)

Anti-Rh Slide Test Serum	One Drop
Blood Specimen (Whole Blood)	Two Drops

Mix with applicator stick. Spread mixture over most of slide. Tilt and rotate slide. Observe for macroscopic clumping.

INTERPRETATION	
Rh Positive	Clumping Within 2 Minutes
Rh Negative	No Clumping Within 2 Minutes

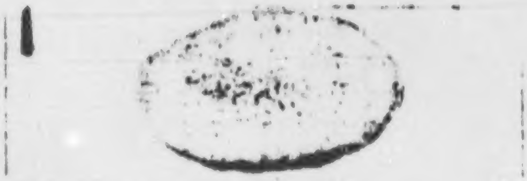
NOTE: Known Rh positive and Rh negative cells should be tested simultaneously as controls.

STEP 1. Place one drop of anti-Rh slide test serum on a slide, warmed on a view box to at least 37°C. but not over 45°C.

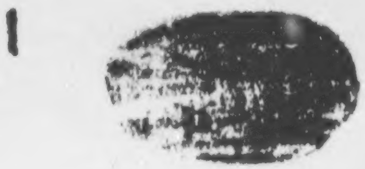
STEP 2. Add two drops of the blood specimen to the serum on the slide.

STEP 3. Mix with a clean applicator stick. Spread the mixture on the slide.

STEP 4. Tilt the view box back and forth and observe for clumping within 2 minutes.



STEP 5. Rh positive.



STEP 6. Rh negative.

Figure 5-1. Slide Method-Albumin Active Rh Antibodies.

5. Never use saline suspensions of cells when testing with slide test serum. This is very important.
6. Follow the manufacturers' directions for the use of their serum if it differs from the above technique.
7. Always make sure that all glassware is properly labeled.
8. Blood to be tested should be fresh.
9. Causes of false positive reactions include—
   - *a.* Using anti-Rh serum containing other than anti-Rh antibody.
   - *b.* Observing for too long a period on viewbox (drying).
   - *c.* Nonspecific agglutination due to chemical contamination of the glassware or bacterial contamination of the antiserum or specimen.
   - *d.* Rouleaux formation.
   - *e.* Sedimentation of erythrocytes which may be erroneously interpreted as agglutination.
   - *f.* Coagulation from excessive heating.
10. Causes of false negative reactions include—
    - *a.* Insufficient incubation time.
    - *b.* Hemolyzed cells.
    - *c.* Blood specimens which have been retained too long after being drawn.
    - *d.* Cell suspension too weak.
    - *e.* Insufficient mixing.
    - *f.* Failure to heat slide.
    - *g.* Outdated or contaminated serum.
    - *h.* Weak typing serum.
    - *i.* Excessive concentration of potassium oxalate.
11. Rh typing serums (especially each new lot of serum) should be checked occasionally by utilizing controls as follows:
    - *a.* Auto-agglutinin control consisting of albumin plus patient's whole blood.
    - *b.* Positive control consisting of typing serum plus known Rh positive blood.
    - *c.* Negative control consisting of typing serum plus known Rh negative blood.

High Protein Tube Test Method of Rh Typing

The test tube technique may be performed routinely on all blood specimens or it may be employed as a procedure to confirm slide test results. Slide test serum is used to insure a high protein concentration in the reaction mixture.

APPARATUS:

Test tubes, Kahn.
Applicator stick, wood.

REAGENTS:

Typing serum, anti-Rh (anti-D (Rh_o) or other, depending upon the antigen of interest).
Normal saline, 0.85 percent.

TECHNIQUE:

1. Select a clean, scratch-free test tube and label it with the patient's name or code number.
2. Place into the tube one drop of anti-Rh typing serum.
3. Dip an applicator stick into blood cells from a clot of whole blood and transfer the erythrocytes adhering to the applicator stick to the typing serum in the test tube. This should result in approximately 2 percent suspension.
4. Mix the contents of the tube and incubate in a 37° C. water bath for 10 minutes. Centrifuge at 1,000 r.p.m. to loosely pack the cells.
5. Remove the tube from the centrifuge and add one drop of normal saline. Twirl the tube between thumb and fingers until cells are loosened from the bottom of the tube.
6. Holding the tube over an illuminated ground glass surface, tilt it so that the liquid contents will flow up the side about 1 inch. Observe for agglutination. This technique is summarized in table 5–4, and the procedure is illustrated in figure 5–2① and 5–2②.

INTERPRETATION:

Rh positive cells will be clumped or remain in the original button which was loosened by twirling. Rh negative cells will form a smooth, even suspension. This interpretation can be applied to the presence or absence of any Rh

Table 5-4. High Protein Tube Test Method of Rh Typing

Clean, Scratch-free Test
Tube, Properly Labeled

Anti-Rh Typing Serum	One Drop
Blood Cells (Whole Blood)	Quantity Adhering to Applicator Stick

Mix contents of tube. Incubate at 37° C. for 10 minutes.
*Centrifuge to loosely pack the cells.

Normal Saline	One Drop

Gently resuspend cells and observe for clumping.

INTERPRETATION	
Rh Positive	Clumping
Rh Negative	No Clumping

NOTE: Known Rh positve and Rh negative cells should be tested simultaneously as controls.
* Time dependent on manufactures instructions.

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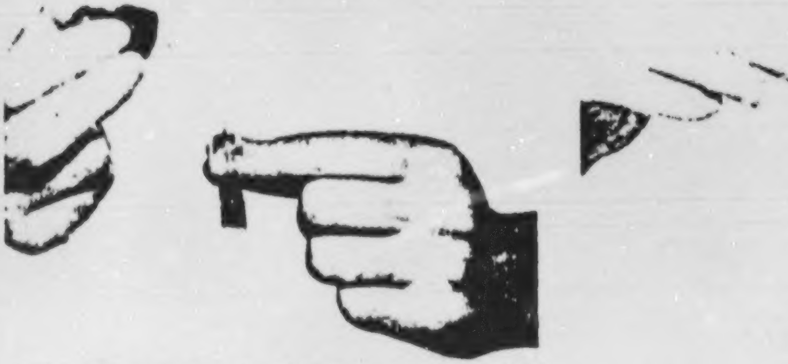
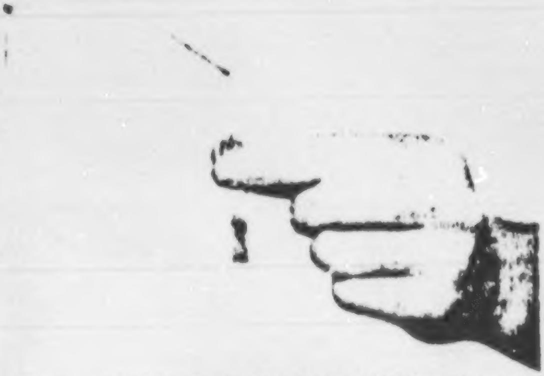
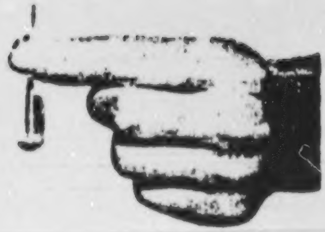


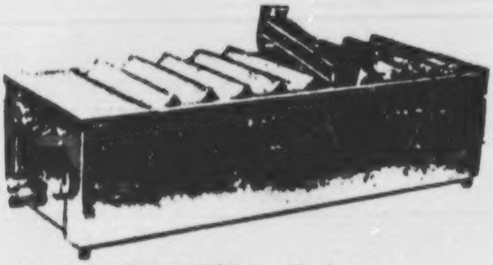
STEP 1. Place one drop of anti-Rh typing serum in a clean labeled test tube.



STEP 2. Dip an applicator stick into the blood specimen and transfer the adhering cells to the test tube.



STEP 3. Mix the cells and serum.



STEP 4. Incubate at 37°C. for 10 minutes.

Figure 5-2①. High protein tube test method of Rh typing.

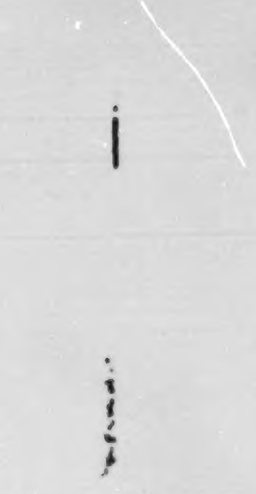
antigen depending on which specific antiserum is employed in the test. All presumptively D (Rh_o) negative tests should be incubated for an additional 50 minutes in a 37° C. water bath and converted to the Coombs test for the detection of the weakly reacting D^u factor.

DISCUSSION:

1. Always follow the manufacturers' directions for using their typing serum.
2. Fresh cells should always be used in testing.
3. Make sure that all tubes are correctly labeled.
4. Examine all apparent negative reactions with the aid of a hand lens or magnifying mirror.
5. Causes of false negative reactions include—
   - *a.* Weak typing sera.
   - *b.* Insufficient incubation time.
   - *c.* Addition of saline at the improper time.
   - *d.* Vigorous agitation of the tube and its contents before reading.
   - *e.* Hemolyzed cells.

ADAMS SERO-FUGE

STEP 5. Centrifuge the tube to loosely pack the cells.



STEP 7. Rh positive.

STEP 6. Add one drop of normal saline, gently resuspend the cells, and observe for clumping.

STEP 8. Rh negative.

Figure 5-2②—continued.

f. Blood specimens which have been retained too long after being drawn.

g. Outdated or contaminated serum.

6. Causes of false positive reactions include—

a. Excessive centrifugation.

b. Misinterpretation of the appearance of the cell mass.

c. Excessive drying prior to reading.

d. Rouleaux formation.

e. Use of chunks of clotted blood.

f. Use of anti-Rh serum containing other than anti-Rh antibody.

g. Nonspecific agglutination due to chemical contamination of the glassware or bacterial contamination of typing serum or specimen.

h. Coagulation from excessive heating.

7. Rh typing sera (especially each new lot of serum) should be checked occasionally by utilizing controls as follows:
    a. Auto-agglutinin control consisting of albumin and patient's whole blood.
    b. Positive control consisting of typing serum plus known Rh positive cells.
    c. Negative control consisting of typing serum plus known Rh negative cells.

SALINE TUBE TEST METHOD OF Rh TYPING

At times, especially when dealing with the blood of infants, the laboratory technician is required to test for Rh antigens with cells suspended in saline. This method is to be used only with anti-Rh typing serum bearing the label statement "For Saline Tube Test."

APPARATUS:

Test tubes, Kahn.
Medicine droppers.

REAGENTS:

Saline agglutinating anti-Rh serum (anti-D (Rh$_o$) or other depending upon the antigen of interest).
Normal saline, 0.85 percent.

TECHNIQUE:

1. Select a clean, scratch-free test tube and label it with the patient's name or code number.
2. Place into the tube one drop of saline agglutinating anti-Rh serum.
3. Add one drop of a 2–4 percent suspension of patient's blood cells in saline. Cells must be saline washed and are best tested as fresh as possible. The saline cell suspension must be used on the day it is prepared.
4. Shake the tube to mix and allow it to stand at 37° C. for 1 hour.
5. Agitate the tube and contents gently and then centrifuge lightly (400–1,000 r.p.m.) for 1 minute.
6. Hold the test tube almost horizontal over an illuminated surface. Roll the tube between the thumb and fingers to dislodge the button of cells from the bottom of the tube. Observe for agglutination. This technique is summarized in table 5-5 and the procedure is illustrated in figure 5-3① and 5-3②.

Table 5-6. Saline Tube Test Method of Rh Typing

Clean, Scratch-free, Properly Labeled Test Tube

Saline Agglutinating Rh Typing Serum	One Drop
Blood Cells, 2-4 Percent Suspension	One Drop

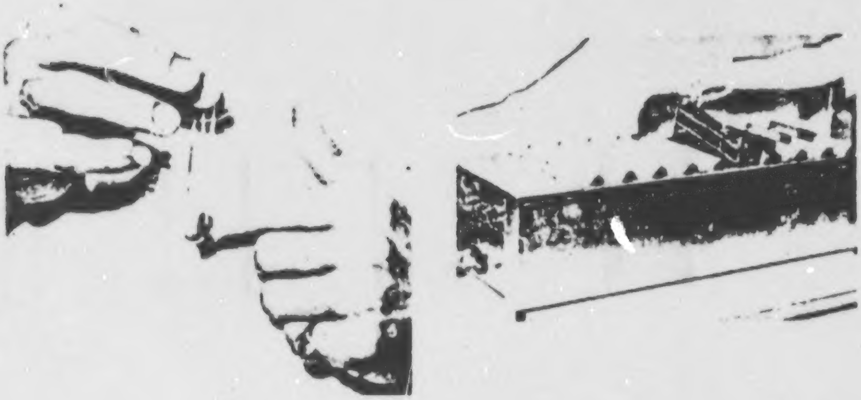
Shake the tube to mix. Incubate at 37°C. for 1 hour. Resuspend cells and centrifuge at 500-1000 rpm for 1 minute. Gently dislodge the button of cells and observe for clumping.

INTERPRETATION	
Rh Positive	Clumping
Rh Negative	No Clumping.

NOTE: Known Rh positive and Rh negative cells should be tested simultaneously as controls.

STEP 1. Place one drop of saline agglutinating Rh typing serum in a clean test tube.

STEP 2. Add one drop of a 2-4 percent suspension of patient's cells.



STEP 3. Gently shake the tube.

STEP 4. Incubate at 37° C. for 1 hour.

Figure 5-3①. Saline tube test method of rh typing.

INTERPRETATION:

Agglutination, if present, is usually grossly visible. Any degree of agglutination indicates a positive reaction. Macroscopic readings may be confirmed by microscopic observation. Excessive shaking of the tubes should be avoided since it may disrupt agglutination and cause false negative readings. This interpretation can be applied to the presence or absence of any Rh antigen depending on which specific antiserum is employed in the test.

Control tests with known positive and negative cells are essential in this procedure and should be run concurrently with the unknown specimen.

All presumptive D (Rh$_o$) negative bloods should be further tested for the weakly reacting Rh$_o$ variant D^u factor.

DISCUSSION:

1. Always follow the manufacturers' directions for using their typing serum.
2. Fresh cells should always be used in testing.

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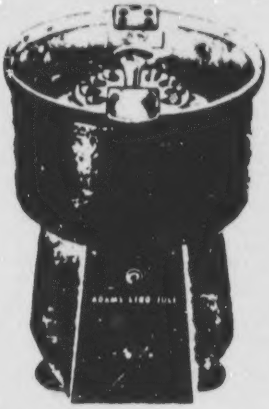
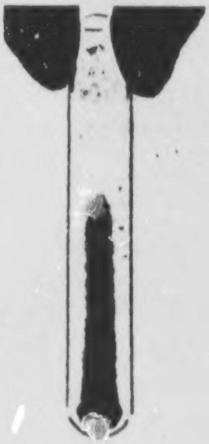


STEP 5. Centrifuge the tube to loosely pack the cells.

STEP 6. Gently dislodge the button of cells and observe for clumping.

STEP 7. Rh positive.



STEP 8. Rh negative.

Figure 5-3②—continued

3. Be sure that all tubes are correctly labeled.
4. Examine all apparent negatives with the aid of a hand lens or magnifying mirror.
5. Causes of false negative reactions include—
   - *a.* Weak typing sera.
   - *b.* Insufficient incubation time.
   - *c.* Vigorous agitation of the tube and its contents before reading.
   - *d.* Hemolyzed cells.
   - *e.* Blood specimens which have been retained too long after being drawn.
   - *f.* Outdated or contaminated serum.
6. Causes of false positive reactions include—
   - *a.* Excessive centrifugation.
   - *b.* Misinterpretation of the appearance of the cell mass.
   - *c.* Excessive drying prior to reading.
   - *d.* Rouleaux formation.
   - *e.* Use of anti-Rh serum containing other than anti-Rh antibody.

f. Nonspecific agglutination due to chemical contamination of the glassware or bacterial contamination of typing serum or specimen.

g. Coagulation from excessive heating.

7. Saline agglutinating Rh typing serum is more unstable than slide test typing serum and, therefore, must be checked regularly by utilizing controls as follows:

   a. Positive control consisting of typing serum plus known Rh positive cells.

   b. Negative control consisting of typing serum plus known Rh negative cells.

D^u FACTOR AND OTHER Rh VARIANTS

Several variants of the six basic Rh genes have been discovered. They include C^w, C^v, C^u, C^x, E^u, D^u, and others. These are referred to as "intermediate genes." The two variants that are best established and of importance in clinical procedures are C^w, a variant of C, and D^u a variant of D. Sensitization to C^w has occurred with subsequent hemolytic transfusion reactions or erythroblastosis. The antigen, however, has been identified in only about 1 percent of the population and, therefore, reactions are relatively rare.

The D^u variant of D has been designated as "weak D or weak Rh_o." This is because it is usually not clearly demonstrable in routine Rh tests. In the Caucasian race, D^u is commonly found in gene combination with C or E. It has been demonstrated that D^u positive blood may immunize the Rh negative patient resulting in the formation of anti-D antibodies. For this reason, it is important to exclude D^u individuals from the Rh negative blood donor list.

Method for Demonstrating the D^u Factor

Blood which is D^u positive will appear to be type D (Rh_o) negative when routine Rh testing procedures are employed. For this reason, antihuman globulin (Coombs serum) must be used to demonstrate the presence or absence of the D^u factor. Antiserum containing the anti-D (Rh_o) antibody is used to "coat" the cells to be tested. This procedure is similar to the indirect Coombs test which will be discussed in detail in chapter 7.

APPARATUS:

Test tubes, Kahn.

Medicine droppers.

REAGENTS:

Anti-D (Rh_o) slide (modified tube) serum.

Antihuman globulin (Coombs) serum.

Normal saline, 0.85 percent.

TECHNIQUE:

In all instances manufacturer's instructions must be rigidly followed.

1. When no agglutination occurs with the patient's cells, plus Anti-D (Rh_o) slide test serum after 10 minutes in a 37° C. water bath and centrifugation at 1,000 r.p.m. for 2 minutes, incubate the tubes for an additional 15 minutes at 37° C.
2. After incubation, remove the tubes from the water bath and wash the cells four times in 0.85 percent saline. Use fresh saline for each wash. Decant the supernate after the last washing, removing as much of the saline as possible.
3. Add one drop of antihuman globulin (Coombs) serum.
4. Centrifuge the tube at 1,000 r.p.m. to loosely pack the cells.
5. Resuspend the packed cells by gentle agitation and examine for agglutination.
6. Readings may be confirmed, if desired, with the aid of a small hand lens or a magnifying mirror. This technique is summarized in table 5-6 and illustrated in figure 5-4① and 5-4②.

INTERPRETATION:

Agglutination indicates that the blood is of the D^u variety. Such bloods should be classified as Rh positive if from a donor and Rh negative if from a recipient for transfusion. Anti-D (Rh_o) slide test serum used for the detection of the Rh_o variant (D^u) factor must be highly specific for the D (Rh_o) factor. Anti-A agglutinins and anti-C (rh') antibody are occasional contaminants in anti-D (Rh_o) serum. With groups A cde and any group with Cde bloods, such sera will give results which may

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Table 5-6. Method for Demonstrating the D^u Factor

Three Clean, Properly Labeled Test Tubes	Test	Negative Control	Positive Control
Processing of Tubes	Tube containing cells exhibiting no reaction in anti-D ($\overline{Rh_o}$) typing serum after 10 minutes at 37° C. and centrifuge to loosely pack	Two Drops Patient's Cells, 2 Percent Suspension One Drop Bovine Albumin, 22 Percent	Two Drops D (Rh_o) Positive Cells, 2 Percent Suspension One Drop anti-D (Rh_o) Slide Test Serum
	Incubate additional 15 minutes at 37° C.	Incubate for 15 minutes at 37° C.	Incubate for 15 minutes at 37° C.

Wash cells in all tubes four times in saline. Decant saline completely after last wash.

Antihuman Globulin Serum	Two Drops	Two Drops	Two Drops

Centrifuge at 500 rpm for 1 minute. Gently dislodge cells and examine for agglutination.

INTERPRETATION			
D^u Positive	Clumping	No Clumping	Clumping
D^u Negative	No Clumping	No Clumping	Clumping

be mistaken for Rh_o variant (D^u) positive reactions. To avoid such errors, recheck any bloods found to be D^u positive with an antiserum of known specificity for D^u testing. If this special antiserum is not available, use two or more anti-D (Rh_o) sera of different manufacturers.

Negative and Positive Controls

To assure maximum test validity, controls should be run along with the test specimens. These controls are as follows:

Negative Control. Place two drops of the patient's 2 percent cell suspension into a tube marked "Negative control." Add one drop of bovine albumin (22 or 30 percent) and mix well. Incubate the tube for 15 minutes at 37° C. and process in a manner similar to that applied to the test specimen.

Positive Control. Place two drops of D (Rh_o) positive 2 percent cell suspension in a test tube marked "Positive control." Add one drop of anti-D (Rh_o) slide test serum and mix. Incubate the tube for 15 minutes at 37° C. and process in a manner similar to that applied to the negative control and the test specimen.

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STEP 1. Incubate the tube giving a negative reaction in the tube test for $D(Rh_0)$, for 50 additional minutes at 37° C.

STEP 2. Wash the cells four times in saline. Decant saline completely after last wash.

STEP 3. Add one drop of antihuman globulin serum.

STEP 4. Allow the tube to stand at room temperature for 15 minutes.

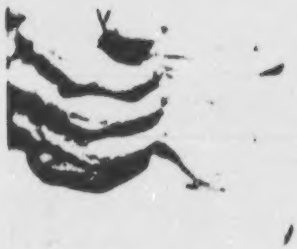
Figure 5-4①. Method for demonstrating the D^u factor.

The direct Coombs test should be done as a control in parallel with the positive and negative controls.

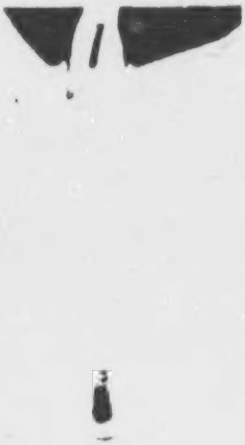
DISCUSSION:

1. Always follow the manufacturers' directions for using their typing serum or antihuman globulin.
2. To avoid false negative reactions, washing of the cells must be adequate to remove all serum proteins.
3. Fresh cells should always be used in testing.
4. Make sure that all tubes are correctly labeled.
5. Causes of false negative reactions include—
   - *a.* Insufficient incubation time.
   - *b.* Diluting cells excessively after washing.
   - *c.* Weak anti-D (Rh_0) and Coombs serum.
   - *d.* Insufficient concentration of test cells.

STEP 5. Centrifuge the tube to loosely pack the cells.



STEP 6. Gently dislodge the button of cells and examine for clumping.



STEP 7. D^u positive.

STEP 8. D^u negative.

*Figure 5-4*③—continued.

e. Incubation temperature too high or too low.

f. Improper washing.

g. Vigorous agitation of the tube and its contents before reading.

h. Hemolyzed cells.

i. Blood specimens which have been retained too long after being drawn.

j. Outdated or contaminated typing and or Coombs serum.

6. Causes of false positive reactions include—

a. Use of anti-D (Rh_o) serum containing other than anti-Rh antibody.

b. Excessive centrifugation.

c. Rouleaux formation.

d. Drying.

e. Blood clots.

f. Misinterpretation of the appearance of the cell mass.

g. Nonspecific agglutination due to chemical contamination of the glassware or bacterial contamination of typing serum or specimen.

h. Coagulation from excessive heating.

i. A positive Dc.

Rh TYPING AND ANTIBODY COATED ERYTHROCYTES

In certain instances the blood bank laboratory may be requested to perform Rh studies on erythroblastotic infants who have been classified as Rh negative. More careful testing may prove these cases to be Rh positive babies in whom the surfaces of the erythrocytes are so heavily or completely coated with blocking antibodies that there is no surface left for the anti-D antibodies to cause agglutination.

The failure to obtain agglutination with potent anti-D serum when the direct anti-human globulin test (ch 7) is positive should make the technician suspect completely blocked red cell surfaces until subsequent tests have proved it otherwise. In such instances the infant's serum should be tested against a panel of group O, Rh positive, and group O, Rh negative cells. Agglutination of all the group O cells that contain factor D and failure to agglutinate the Rh negative cells will be proof that the baby is Rh positive.

HEAT ELUTION PROCEDURE FOR DEMONSTRATION OF ABSORBED ANTIBODY

In an attempt to identify the antibodies coating the newborn's red cells, the following technique is recommended:

TECHNIQUE:

1. Prepare a 25 to 50 percent suspension of red cells in saline and wash at least three times with large quantities of saline.
2. Pack the cell mass and completely remove all of the last wash.
3. To one volume of packed cells add one volume of fresh saline and resuspend.
4. Place the mixture in a 56° C. water bath for 10 minutes, agitating frequently.
5. Remove and immediately centrifuge at high speed in centrifuge cups heated to 56° C.
6. Remove the hemoglobin-tinted supernatant fluid and test for antibodies in the same fashion as serum.

By utilizing this heat elution technique, the true nature of the fetal cells will be determined and at the same time the maternal antibody coating these cells is isolated and can subsequently be identified definitively.

CRITERIA FOR ANTI-Rh TYPING SERA

Rh typing procedures yield valid results only if potent anti-Rh typing serum is employed. The sera used must meet the requirements established by the National Institutes of Health (Division of Biologic Standards). In order to be considered suitable, anti-Rh typing serum must be clearly identified as to specificity and method of use. The serum must also pass tests for specificity, specific agglutinin titer, and avidity.

Specificity

Specificity of the antiserum is confirmed by demonstrating the absence of all antibodies other than those indicated on the container label. Serum, prepared by laboratories licensed by the National Institutes of Health (Division of Biologic Standards), is tested before release to insure its freedom from hemolysins, anti-A and anti-B agglutinins, and other types of anti-Rh antibodies, etc. Anti-D (Rh$_o$) typing serum will agglutinate all blood cells containing the D (Rh$_o$) antigen. Anti-D (Rh$_o$) serum will not agglutinate other cells, regardless of the antigens present, i.e., cells such as O C'de, A cde, B cdE, etc. Similarly, anti-C (rh') typing sera will agglutinate only cells containing C (rh').

Specific Agglutinin Titer

Properly prepared Rh typing serum should be rich in specific antibodies. Anti-D (Rh$_o$) typing serum, to be satisfactory, should show a minimum titer of 1:32 against cells of types CDe and cDE. Anti-CD (Rh'$_o$) typing serum should exhibit a titer of 1:32 or more against cells of types CDe or cDE. These titers are very conservative values and the blood bank technician will frequently encounter values far in excess of those stated above.

Avidity

Avidity refers to the speed and intensity of reaction. Antisera employed in tube tests must be free of a tendency to promote prozone phenomena and exhibit a strong reaction under the prescribed conditions of use. Antisera labeled "For Saline Tube Test" must be carefully and continually watched to be sure that they persist in meeting the above qualifications since unexpected deterioration may occur.

Antisera which are labeled "For Slide Test" must show prompt and intense reactions when used in this procedure. All slide test sera should be tested on a slide against two times the volume of a 40 percent cell suspension in protein containing medium. Under these conditions, reactive cells should begin to show a macroscopic reaction in 60 seconds or less. The reaction should proceed within 2 minutes to the formation of cell agglomerates, some of which are 1 square mm. or more in surface area.

SOURCES OF ERROR IN Rh TYPING

In order to obtain valid results in Rh typing, the technician must pay close attention to detail. Some of the most common sources of error are discussed below.

Weak or Nonstandardized Anti-Rh Typing Sera

It is extremely important that sera prepared according to National Institutes of Health (Division of Biologic Standards) standards be used. Antisera labeled "For Saline Tube Test" even though satisfactory initially, have a distinct tendency to deteriorate unexpectedly. These antisera, therefore, must always be used in conjunction with known positive and negative controls. If saline test antisera are stored in the frozen state, deterioration will be minimized.

Bacterial Contamination of Serum

Contamination of serum by bacteria may cause the formation of bacteriogenic panagglutinins which agglutinate all cells or may weaken the serum by destroying the desired agglutinins. It is essential that clean technique be used in opening the container. In use, the dropper-cap assembly should be protected from contamination and returned promptly to the bottle. The dropper is the only item which should be placed into the container of anti-Rh typing serum. Rh antiserum should be stored in the refrigerator at all times when it is not being used. This will retard the growth of bacteria which do gain access to the serum. The sodium azide added to the serum as a preservative will protect against many but not all possible contaminants. It cannot take the place of the careful attention which must be given to the avoidance of contamination.

Chemical and Biochemical Deterioration

Antiserum should be kept from contact with traces of chemicals. Such chemical contamination may occur when using improperly cleaned pipettes, slides, or test tubes. Typing sera intended for the saline test are much less stable than those designated for the slide test. Proper refrigeration will retard deterioration.

Improper Storage of Anti-Rh Typing Serum

Anti-Rh typing serum for slide testing should be stored in the refrigerator at 2°–10° C. when not being used. Lower temperatures may be used for long-term storage. Frozen serum, however, must be allowed to return to the liquid state completely before using. The serum should be well mixed by repeated squeezing of the dropper bulb. Typing serum for the saline tube test may be stored in the refrigerator as described above but the relative instability of this type of serum makes it very desirable that storage in the frozen state be routinely employed. Such low temperature storage retards antibody deterioration and minimizes growth of chance bacterial contaminats.

Misunderstanding of the Specificity of the Serum Employed

The degree of specificity of a particular typing serum must be properly understood. For example, the occasional individual of type Cde would appear D (Rh_o) negative if tested with anti-D (Rh_o) serum but would exhibit a positive reaction if tested with anti-CD (Rh'_o) serum. An individual of type Cde, if a blood recipient or maternity case, is just as likely to be immunized against the D (Rh_o) antigen as a type cde individual and should, therfore, be properly classified as Rh negative. The same individual, if a blood donor, must be removed from the Rh negative donor list because of the possibility that administration of his blood might react with anti-C (rh') antibodies present in the recipient's serum.

Misunderstanding of the Variety of Antibody in the Serum Employed

It is extremely important that the antiserum manufacturer's instructions be closely followed. A serum labeled "For Saline Tube Test" should be used only with a saline suspension of cells. A serum designated "For Slide Test" should not be used with cells suspended in saline. If serum is employed in any manner which deviates from the method prescribed by the manufacturer, the technician should take every precaution to be sure that the variations of procedure are permissible.

Improper Blood Specimen

Blood specimens should be properly collected, accurately identified, placed promptly in the refrigerator, and tested as soon as possible. If the specimen is not obtained at the laboratory, the physician, nurse, or technician collecting the specimen, should be aware of the complete and proper procedure employed. Specimens unidentified or poorly identified, improperly collected, hemolyzed, or more than 2 day's old should be rejected and fresh specimens obtained. A specimen diluted by saline, citrate, or oxalate solutions may show weak or no reactions in the slide test.

Improper Cell Suspensions

Two percent suspensions of cells in saline must be used on the day they are prepared and preferably immediately. Saline suspensions may deteriorate in 2 or 3 hours, especially in hot weather. Suspensions not used immediately should be stored in the refrigerator and discarded at the end of the day.

The whole blood (approximately 50 percent cell suspension) used in the slide test must be in a final concentration of at least 20 percent in the mixture on the slide. A lower concentration of cells may result in weak or even negative appearing results. Insufficient cells might be added if the specimen is low in erythrocytes (anemia) or if inadequate numbers of cells are transferred from blood clots.

Temperature During Testing

Rh antibodies are agglutinins which react with maximum intensity at a temperature of 37°–45° C. Proper temperature is attained in test tube procedures by using an incubator or water bath. For the slide test, the desirable temperature is 37°–47° C. If the temperature is too low, weak or false negative reactions may occur. A temperature which is too high also tends to retard the rate of reaction but is primarily objectionable because of the misleading effects of excessive desiccation. Overheating should be especially avoided with Rh negative cells. It is important to employ a thermometer to be sure that the viewing box employed in the slide test is at the proper temperature. Most Rh viewing boxes require considerable time to reach the proper temperature.

The illuminated surface of the box may be unevenly heated and some areas may be too warm while other portions will be excessively cold. Room temperature, of course, will influence the temperature of the viewing surfaces.

Excessive Agitation of Test Tube Mixtures After Centrifugation

In test tube procedures, it is necessary to dislodge the button of sedimented cells from the bottom of the tube prior to reading. Agitation at this point can easily be overly vigorous and disturb agglutination resulting in a false negative interpretation. This is probably the most common source of error in saline tube tests. The preferred technique of dislodging the button of cells is to first hold the tube nearly horizontal over an illuminated surface. Rotate the tube on its own axis and gently dislodge the button of cellular sediment. The preliminary centrifuging should be sufficient to produce a definite button, but not so great that it compels the technician to shake the tube excessively.

Doubtful or Weak Slide Reactions

A general discussion of rouleaux formation (pseudoagglutination) was presented in chapter 2. Specifically, rouleaux formation in Rh typing

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may be caused by an excessively high temperature of the viewing box (over 47° C.) or by waiting too long (over 2 or 3 minutes) before taking final readings. Rouleaux appears more commonly when testing oxalated blood specimens and may also occur with the use of excessive heparin. Rouleaux formation is least likely to be observed with clotted blood specimens. The elimination of rouleaux was discussed in chapter 2.

Occasionally, blood coagulation may appear during the slide test if oxalated or heparinized specimens are used. This condition may be suspected when translucent, whitish, shiny masses or strands develop in the test mixture. This source of error may be avoided by employing larger amounts of anticoagulant. This difficulty is also avoided by using clotted blood provided that sufficient time is permitted to elapse between venipuncture and testing so that coagulation will be complete.

Sometimes the patient's own serum may cause false positive reactions. The possibility of this occurrence may be suspected in blood dyscrasias, hyperproteinemia, etc. Using the saline tube technique will avoid this error. An alternate method is to repeat the slide test with the patient's cells suspended, not in his own serum, but in other protein containing media such as normal serum of the same group, group AB serum, or 20-30 percent bovine albumin.

Failure to Use Positive and Negative Controls

All of the methods of Rh typing require close and careful attention to procedural details. It is strongly recommended that known positive and known negative cells be employed as controls. This is especially important when tests are performed only occasionally or with antiserum designated for use with saline suspensions of erthrocytes.

Rh Typing in the Neonatal Period

Rh typing during the neonatal period often results in doubtful test reactions. Prior transfusion of blood to the infant may have introduced so many donor cells that the test determines the Rh factor on the donor's erythrocytes rather than the child's. This particular source of error will most likely result in false negative reactions. The transfused erythrocytes may persist in the infant's circulation up to 3 months after transfusion.

Blocking antibodies passing through the placental barrier from the mother may have been adsorbed onto the child's red cells. The child's erythrocytes, if coated by blocking antibodies, may fail to clump when testing with saline tube test antiserum of the proper specificity. The infant's coated cells may behave in another way; they may agglutinate spontaneously when tested in the slide method using normal adult serum. Since adult human serum is a component of anti-Rh typing serum, the positive reaction encountered in the slide test using antibody coated cells is not necessarily specific. Generally speaking, a positive reaction in the neonatal period is more than likely correct and a negative reaction in the first few months of life may or may not be correct. Bovine albumin, which is present in some Rh typing sera, may behave in a manner similar to adult human serum.

Placental cord blood may promote gross pseudoagglutination if Wharton's jelly is inadvertently mixed into the specimen. Blood of the newborn which is to be typed for Rh should be obtained from the umbilical blood vessels by using approved venipuncture technique.

Error in Identification

All blood specimens should be very carefully and completely identified with a label attached to the collecting tube at the time the specimen is obtained. Gummed paper labels or adhesive tape are recommended since complete identifying data can be placed directly upon them.

CHAPTER 7

COOMBS ANTIHUMAN GLOBULIN TESTS

Coombs antihuman globulin tests may be better understood if a brief review of immunological reactions is first considered. The usual reaction encountered in blood banking can be summarized as follows: A specific antibody (hemagglutinin) reacts with red blood cells possessing the corresponding specific antigen agglutinogen). The reaction, as a rule, passes through two major phases. First, the specific antibody reacts with the specific antigen present in the erythrocyte and, as a result, becomes attached to the erythrocyte. Cells in this condition are said to be coated. Secondly, the cells coated with specific antibodies come together with resulting agglutination.

In some immunologic reactions, however, there are instances in which coating of the erythrocytes occurs but spontaneous agglutination does not follow. These particular reactions may be missed by the technician unless special detection methods are employed. Among such special procedures is the use of a particular test serum which promotes visible agglutination of coated erythrocytes and thus demonstrates the occurrence of a previous reaction between the blood cell antigens and antibody. The standard name for this special serum is antihuman serum. It is also known as Coombs serum, Coombs test serum, antiglobulin serum, and antihuman globulin serum. Antihuman serum is prepared from the blood of animals which have been immunized against purified human globulin.

Antibodies vary in relation to their optimum temperature and medium of reactivity. These differences have been discussed in chapters 5 and 6. Antihuman serum is of use in promoting visible agglutination of erythrocytes which have reacted with antibodies of the antiglobulin or albumin active variety. These incomplete univalent antibodies require a reaction between themselves and the antihuman serum for demonstration. This reaction between incomplete antibodies and antihuman serum is utilized in a direct and indirect test procedure. The steps of the direct and indirect test reactions are illustrated in figures 7-1 and 7-2. The uses of these two tests and their procedures are presented in the following material.

DIRECT COOMBS TEST

This test is used to—

1. Test cord bloods to determine "in utero" sensitization.
2. Demonstrate the presence of auto-antibody coating in cases of acquired hemolytic anemia.
3. Determine sensitization due to a transfusion reaction.

Controls

Negative and positive controls should be routinely run with all direct Coombs tests.

1. Negative control.

   Place two drops of a 2 percent suspension of group O, Rh negative cells in a test tube. Add two drops of anti-D (Rh_o) slide test serum.
2. Positive control.

   Place two drops of a 2 percent suspension of group O, Rh positive cells in a test tube. Add two drops of anti-D (Rh_o) slide test serum.

   Incubate the positive and negative controls in a 37° C. water bath for 30 minutes. During this period the Rh positive cells become coated with anti-D antibodies. Wash the cells in the negative and positive control tubes three times in saline. Decant the saline completely after the third wash. The

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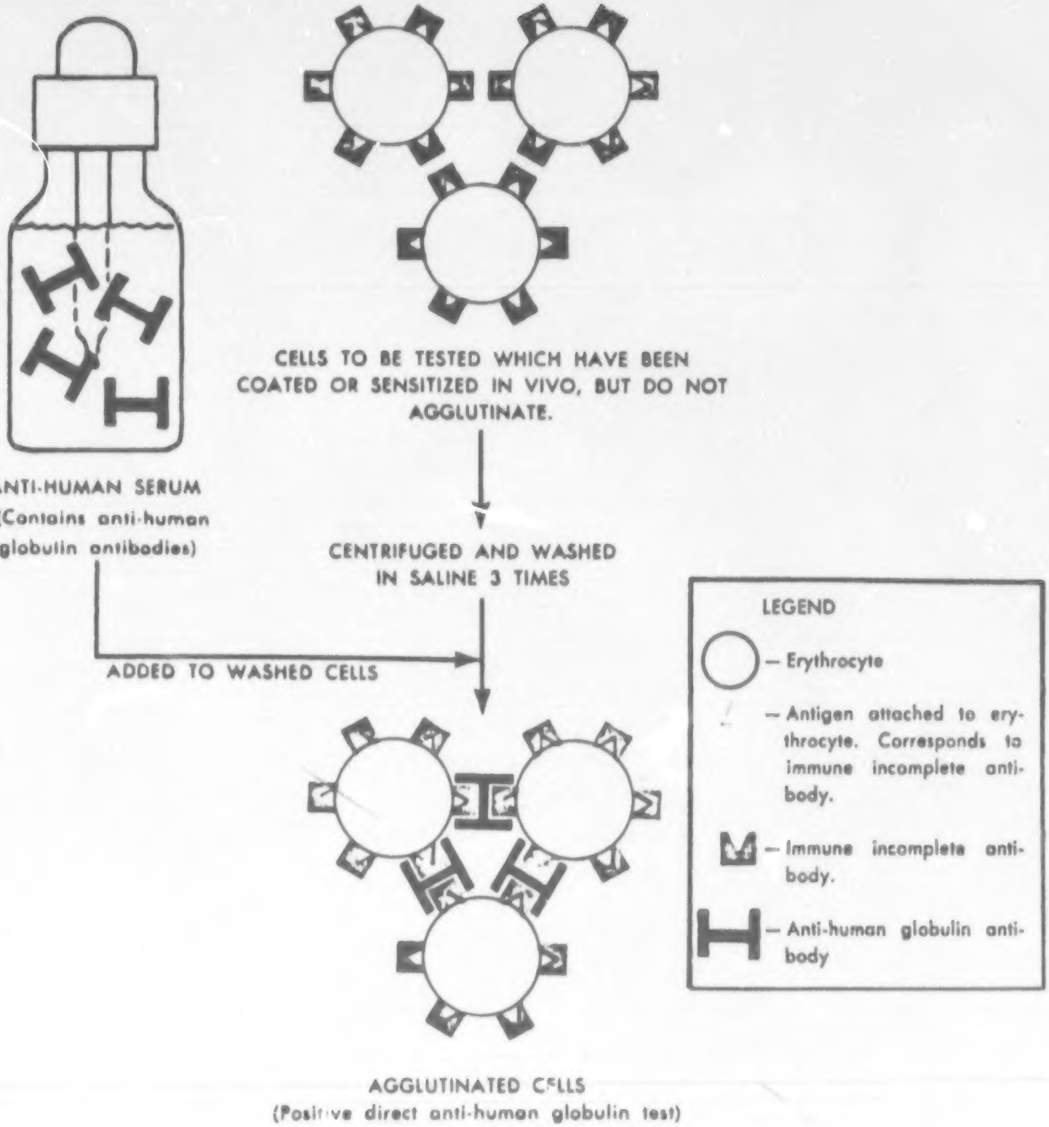

CELLS TO BE TESTED WHICH HAVE BEEN COATED OR SENSITIZED IN VIVO, BUT DO NOT AGGLUTINATE.
ANTI-HUMAN SERUM (Contains anti-human globulin antibodies)
CENTRIFUGED AND WASHED IN SALINE 3 TIMES
ADDED TO WASHED CELLS
LEGEND
— Erythrocyte
— Antigen attached to erythrocyte. Corresponds to immune incomplete antibody.
— Immune incomplete antibody.
— Anti-human globulin antibody
AGGLUTINATED CELLS (Positive direct anti-human globulin test)

Figure 7-1. Direct antihuman globulin test.

controls should be processed from this point in a manner identical to that applied to the unknown.

Test Tube Method

1. Prepare a three or four times washed 2-4 percent suspension of the cells to be tested in saline.
2. Add one drop of antihuman (Coombs) serum to a small (10 x 75mm) or (12 x 75mm) test tube.
3. Add two drops of the cell suspension. Shake to mix.
4. Centrifuge immediately for 1 minute at 1,000 r.p.m.
5. Read and record reaction.

INTERPRETATION:

1. The positive control should exhibit clumping. This indicates that the antihuman serum is functional.
2. The negative control should not show

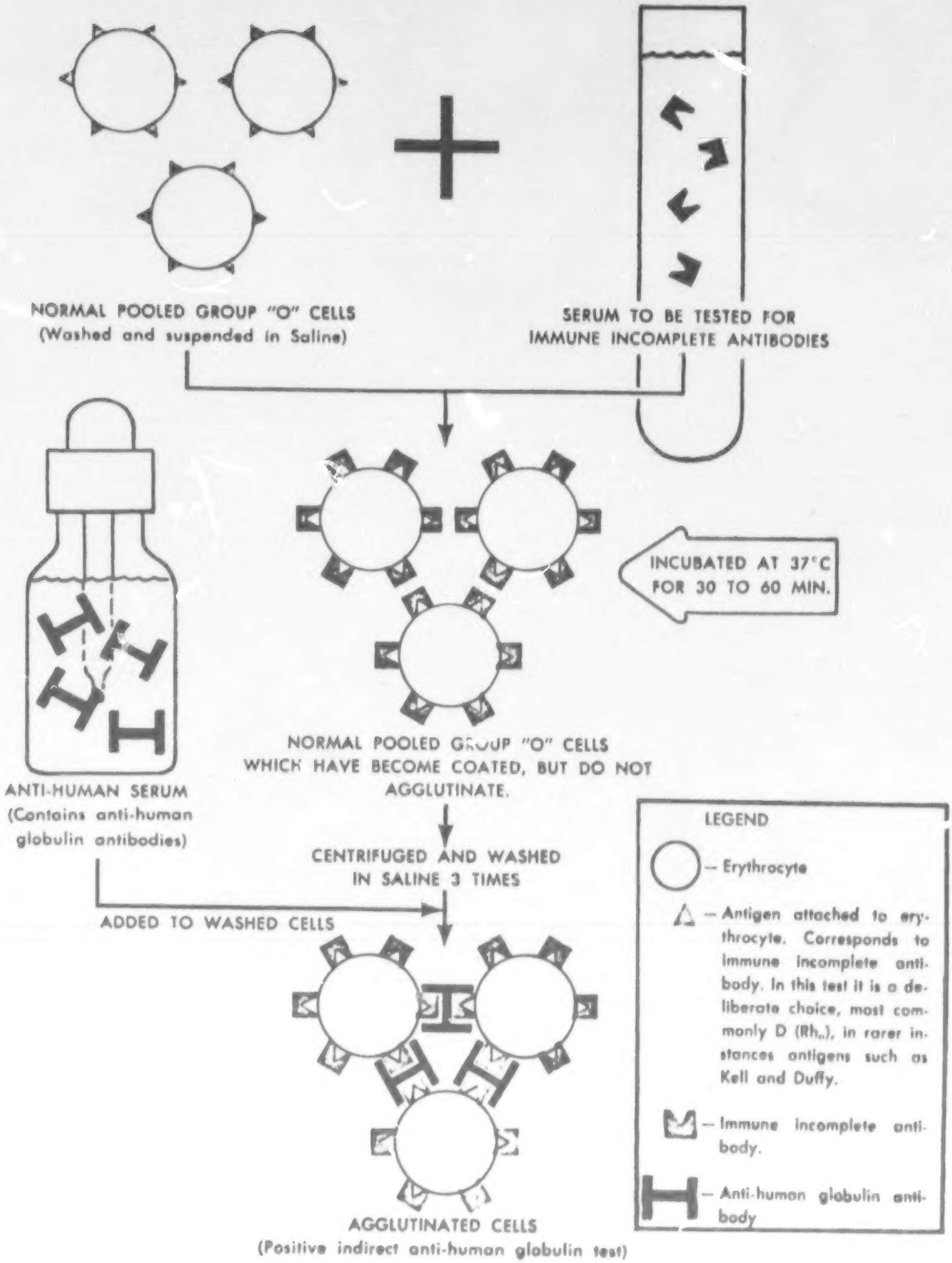

NORMAL POOLED GROUP "O" CELLS
(Washed and suspended in Saline)
SERUM TO BE TESTED FOR
IMMUNE INCOMPLETE ANTIBODIES
INCUBATED AT 37°C
FOR 30 TO 60 MIN.
ANTI-HUMAN SERUM
(Contains anti-human
globulin antibodies)
NORMAL POOLED GROUP "O" CELLS
WHICH HAVE BECOME COATED, BUT DO NOT
AGGLUTINATE.
CENTRIFUGED AND WASHED
IN SALINE 3 TIMES
ADDED TO WASHED CELLS
AGGLUTINATED CELLS
(Positive indirect anti-human globulin test)
LEGEND
— Erythrocyte
— Antigen attached to erythrocyte. Corresponds to immune incomplete antibody. In this test it is a deliberate choice, most commonly D (Rh_o), in rarer instances antigens such as Kell and Duffy.
— Immune incomplete antibody.
— Anti-human globulin antibody

Figure 7-2. Indirect antihuman globulin test.

agglutination. Coating of cells should not have occurred, thus the antihuman serum will not clump the cells. This indicates that the antihuman serum is specific for coated cells only.

3. Coated or sensitized cells will be agglutinated positive reaction. Uncoated will remain unagglutinated negative reaction.

INDIRECT COOMBS TEST

This test is used to—

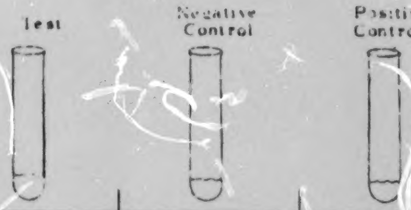
1. Identify unknown antibodies in serum when tested against known test cell panels.
2. Reveal the presence of antibodies in prenatal testing.
3. Detect the presence of the D^u and other cell antigens (Kell, Duffy, Kidd).
4. To reveal incompatibilities in crossmatching.

The indirect test is of value in demonstrating circulating incomplete antibodies present in a patient's serum due to immunization. Using the indirect procedure it is also possible to determine the titer of an incomplete antibody present in a serum. Here, known specific red cells are sensitized to a series of serum dilutions and tested with antihuman serum. A positive reaction represented by agglutination, indicates the presence of such antibodies and a negative reaction indicates the absence of such antibodies.

Cells of unknown or doubtful antigenic content may be exposed to an antiserum known to contain specific antibodies. After the addition of antihuman serum in this variation of the indirect test, a positive reaction indicates the presence of a specific antigen in the cells and a negative reaction indicates the absence of this factor. Certain blood group antigens are thus demonstrated by the use of antihuman serum in conjunction with a specific antiserum.

In preparing blood for use in transfusion, donor's cells and recipient's serum (major side of the crossmatch) are mixed to ascertain if a combination of antigen and antibody exists. A positive reaction after the addition of antihuman serum indicates incompatibility.

Table 7-1. The Direct Antihuman Globulin (Coombs) Test



Tube Designation	Test	Negative Control	Positive Control
Group O, Rh Negative Cells 2 Percent Suspension		Two Drops	
Group O, Rh Positive Cells 2 Percent Suspension			Two Drops
Patient's Blood Cells 2 Percent Suspension	Two Drops		
Anti-D (Rh_o) Slide Test Serum		Two Drops	Two Drops

Incubate at 37°C. for 30 minutes. Wash three times in saline. Decant completely after last wash.

Anti-Human Globulin Serum	Two Drops	Two Drops	Two Drops

Shake the tubes to mix their contents. Incubate at room temperature for 15-30 minutes. Centrifuge at 1000 rpm for 1 minute. Gently resuspend cells and observe for clumping.

INTERPRETATION			
Positive Test	Clumping	Clumping	No Clumping
Negative Test	No Clumping	Clumping	No Clumping

The principle of the indirect test is simple. The test erythrocytes are mixed with antihuman serum after a prior treatment consisting of separation from the original blood specimen, intentional mixing with the patient's serum and washing. The indirect test, then, is used to determine whether coating of the cells has occurred artificially within the test tube.

Controls

1. Negative control.
   Place two drops of a 2 percent suspension of Group O, Rh positive cells in a test tube. Add two drops of normal saline.
2. Positive control.
   Place two drops of a 2 percent suspension of Group O, D^u positive cells in a test tube. Add two drops of anti-D (Rh_o) slide test serum. Positive control may also be a commercial cell reagent.

The controls should be processed concurrently with the unknown test specimen and treated in a manner identical to that applied to the test specimen.

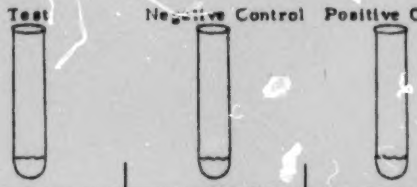
TECHNIQUE:

1. Prepare a three or four times washed 2–4 percent suspension of known cells in saline.
2. Add two drops of unknown serum or desired typing serum to a small (10 x 75mm) or (12 x 75mm) test tube.
3. Add two drops of the cell suspension. Shake to mix.
4. Incubate at 37° C. for 15–30 minutes.
5. Wash three or four times with saline. Decant completely.
6. Add one drop of Coombs serum and mix.
7. Centrifuge immediately for 1 minute at 1,000 r.p.m.
8. Read and record reaction.

Coated or sensitized cells will be agglutinated—positive reaction.

Uncoated cells will remain unagglutinated—negative reaction.

Table 7–2. The Indirect Antihuman Globulin (Coombs) Test



Tube Designation	Test	Negative Control	Positive Control
Group O, Rh Positive Cells 2 Percent Suspension	Two Drops	Two Drops	Two Drops
Patient's Serum	Two Drops		
Anti-D (Rh_o) Slide Test Serum (When test cell is D positive)			Two Drops
Normal Saline		Two Drops	

Mix and incubate at 37°C. for 15-30 minutes. Centrifuge at 500-1000 rpm for 1 minute. Observe for agglutination. If observed, a saline active antibody is present. If not observed, wash cells three times in saline. Decant completely after last wash.

Anti-Human Globulin Serum	Two Drops	Two Drops	Two Drops

Mix and incubate at room temperature for 15 minutes. Centrifuge at 500-1000 rpm for 1 minute. Gently resuspend the cells and observe for clumping.

INTERPRETATION			
Positive Test	Clumping	No Clumping	Clumping
Negative Test	No Clumping	No Clumping	Clumping

DISCUSSION OF COOMBS TEST

1. The manufacturer's instructions accompanying the Coombs reagent must be rigidly followed.
2. Centrifugation should be sufficient to bring the cells together in buttons at the bottom of the tubes. Excessive centrifuging, however, may cause false positive reactions.
3. Fresh cells should always be used in testing.
4. All glassware employed in the antihuman globulin tests must be correctly labeled.
5. Causes of false negative reactions include—
    - *a.* Test cells improperly prepared or inaccurately designated.
    - *b.* Insufficient incubation time.
    - *c.* Hemolyzed cells.
    - *d.* Insufficient washing of cells.
    - *e.* Excessive shaking of tubes prior to reading.
    - *f.* Weak antihuman or typing serum.
    - *g.* Excessively heavy test cell suspensions.
    - *h.* Outdated or contaminated typing or antihuman serum.
6. Causes of false positive reactions include—
    - *a.* Excessive centrifugation.
    - *b.* Using antihuman serum containing other than antihuman globulin antibodies.
    - *c.* Nonspecific agglutination due to bacterial contamination of the antiserum or specimen.
    - *d.* Rouleaux formation.

CRITERIA FOR ANTIHUMAN SERUM

It is essential that antihuman serum be potent and free of nonspecific reactions. All antisera employed in the blood bank laboratory should meet the standards established by the National Institutes of Health (Division of Biologic Standards). In order to be satisfactory, antihuman serum must not exhibit a tendency toward nonspecific reactions as indicated by nonagglutination of various types of normal human red cells mixed with the antihuman serum.

Potency of the serum may be demonstrated in the following manner:

1. Select a test serum of specificity anti-D (Rh_o). Check this test serum for titer under high protein tube test conditions against D (Rh_o) positive cells. This titration will provide control data concerning the potency of this anti-D (Rh_o) serum.
2. Prepare a serial saline dilution comparable to the above and test against sensitized Rh positive cells in saline with the antihuman serum being checked. The antihuman serum should promote obvious agglutination through at least the same range of dilutions as the anti-D (Rh_o) test serum when the latter is employed under high protein conditions. Also, antihuman serum when diluted 1:4 should be as effective as the undiluted antihuman serum. Reactivity of the Coombs reagent against Kell, Duffy and other sensitized cells can be tested in a similar manner.

COLD AGGLUTININS

The presence of cold agglutinins can create difficult problems in the interpretation of antihuman globulin tests. Cold agglutinins are capable of coating erythrocytes as effectively as specific agglutinins and may, therefore, lead to confusing positive results when Coombs tests are performed. Theoretically, blood cells can be washed free of cold agglutinins by using normal saline at 37° C. Practically speaking, however, the removal of all cold agglutinins may be difficult. If these nonspecific antibodies are present, a positive reaction with antihuman serum fails to answer the question of whether the clumping observed is due to cold agglutinins or to specific coating antibodies.

CHAPTER 10

OPERATION OF A BLOOD DONOR CENTER

A complete blood transfusion service includes collection, processing, storage, and administration of human blood and its component parts. The successful maintenance of such a service requires competent personnel who are thoroughly familiar with all phases of its operation. This chapter will present recommended procedures for the efficient operation of part of this service, namely, a blood donor center.

SOURCES OF DONOR BLOOD

Volunteer Donors

Volunteer donors on military bases provide part of the requirement for blood. These donors receive no payment. Many military blood banks, because of their geographic location, are compelled to rely exclusively upon this source of donor blood.

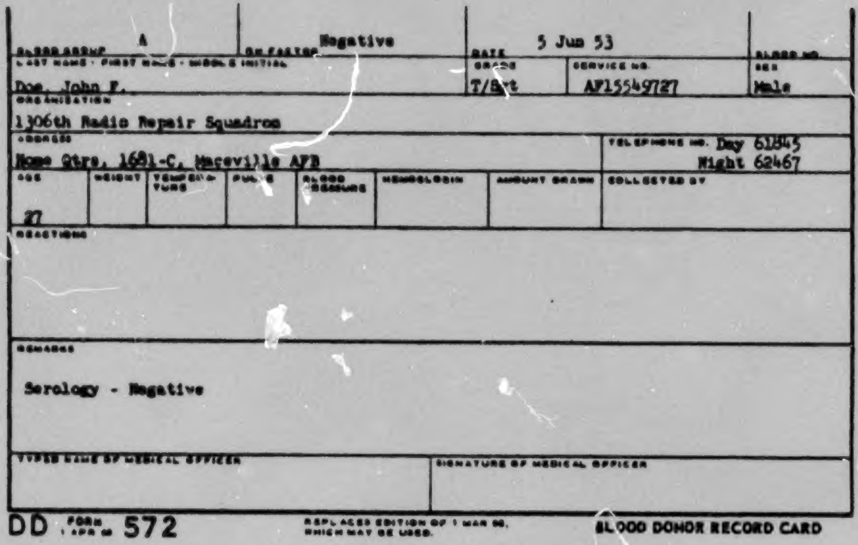
Walking Donor Roster System. All personnel on active duty must have their blood group and Rh type recorded on their DD Form 737 (Immunization Certificate). Also, each medical treatment facility in the military is required to maintain a list, by blood group and Rh type, of all military and civilian personnel who have volunteered to donate blood. Records of all withdrawals of blood from these donors must be kept. DD Form 572 (Blood Donor Record Card), will be used for this purpose. All of the requirements listed above can be met by following a direct and simple course of action. The mandatory grouping and typing of all personnel, along with the maintenance of a volunteer donor roster, can be accomplished by having the blood bank laboratory as a part of the base clearance list. When personnel clear into the base, the blood bank laboratory can check to see if their blood group and Rh type are correct and entered on their records. The information is transcribed to DD Form 572, which is placed in the walking donor file. The prospective donor's duty status and personal desires relevant to giving blood are, of course, placed on the card. The file is broken down by blood group, Rh type, and organization. When personnel clear out of the base, this information is placed in their medical records. If this system were practiced by all bases, the walking blood bank could be maintained with very little effort.

Grouping and typing of personnel, who do not have the above information on file and who are already assigned to a base, require a different approach. A suggested procedure is to have two or three man teams, equipped to perform blood grouping and Rh typing, go to the various organizations on base. Such a team is capable of processing about 75 individuals per hour using finger puncture techniques.

Walking Donor Card Preparation. DD Form 572 (fig. 10-1) will be used for keeping a record of the volunteer blood donors at a particular military activity. The information placed on these cards must be accurate and complete prior to filing. After a person has donated blood, his file card should be placed at the rear of the card file for his particular blood group and Rh factor. Each donation of blood should be indicated on the card along with the exact date and the circumstances surrounding it. An example of a typical entry follows:

Donations

1. 15 July 1954.
   Emergency need. No other donor available with this blood group and Rh factor during time interval required. Patient's doctor, C. E. Smith, approved use of this donor.



BLOOD GROUP A | RH FACTOR Negative | DATE 5 Jun 53 | BLOOD NO.

LAST NAME · FIRST NAME · MIDDLE INITIAL Doe, John F. | GRADE T/Sgt | SERVICE NO. AF15549727 | SEX Male

ORGANIZATION 1306th Radio Repair Squadron

ADDRESS Home Qtrs, 1681-C, Maceville AFB | TELEPHONE NO. Day 61845 Night 62467

AGE 27 | WEIGHT | TEMPERATURE | PULSE | BLOOD PRESSURE | HEMOGLOBIN | AMOUNT DRAWN | COLLECTED BY

REACTIONS

REMARKS

Serology - Negative

TYPED NAME OF MEDICAL OFFICER | SIGNATURE OF MEDICAL OFFICER

DD FORM 1 APR [illegible] 572 REPLACES EDITION OF 1 MAR 50, WHICH MAY BE USED. BLOOD DONOR RECORD CARD

Figure 10-1. A sample of entries on DD Form 572 being used to maintain a volunteer blood donor file.

American Red Cross

The American Red Cross is used as a source of donor blood by some military activities located in Continental U.S. areas having Red Cross regional blood programs. In some cases this is the sole source of donor blood. In others, the Red Cross merely augments the blood requirements of the blood bank.

Civilian Blood Banks

Civilian blood banks licensed by the National Institutes of Health (Division of Biologic Standards), may also furnish blood and/or its components to military blood banks. This blood is either purchased directly or is obtained in exchange for blood from volunteer donors.

Direct Purchase

When whole blood is not available from the sources specified above, it may be purchased directly from individuals. It must be pointed out, however, that the money paid for such blood must come from funds available under the operation and maintenance appropriation of the medical treatment facility.

Note. Aircrews of high performance (jet) or combat aircraft, and persons occupying cockpit positions on an on-call status to perform essential flight duties, will not donate blood. Individuals on flying status assigned to other duties will not be encouraged to donate blood, except in emergencies or unusual circumstances. In no event will personnel perform flying duties for 72 hours following donation of blood. Donors engaged in other hazardous occupations, such as crane operations, diving, or heavy construction projects should not donate blood unless such individuals can be excused from these duties for 12 hours following the donation of blood.

TECHNICIAN-DONOR RELATIONSHIP

In order to maintain an ideal relationship between laboratory blood bank personnel and donors, it is mandatory that the technician treat all donors as he would like to be treated. The following considerations will aid in establishing this desirable relationship:

1. When a donor is contacted by telephone, it must be determined whether he has adequate transportation. If not, transportation to and from the blood donor center should be arranged.
2. If the meal hour follows closely after blood donation, arrangements should be made for the blood donor to be assured an opportunity to obtain a meal.
3. Following blood collection, the donor should be provided with some type of fluid such as fruit juice.

4. The technician should be courteous, patient, tactful, and self-confident in all his procedures.
5. If many donors are present at one time, they should not be kept waiting. Arrangements should be made to have two or three additional blood bank personnel assist in donor blood collection. Vacant rooms on a ward or in surgery can be utilized to speed up the operation of processing donors.
6. The blood collecting procedure should be made as painless for the donor as possible. The technician should make every effort to avoid producing hematomas or other injuries.
7. The technician should let the donor know that his contribution of giving blood is a generous act and very much appreciated. If his blood is to be used for a known patient, it might be feasible for the technician to relate the circumstances which account for the need for the blood.
8. Commanding officers and supervisors of all personnel should be made aware of the necessity for their cooperation and assistance in a blood bank program. Likewise, personal appreciation should be expressed to them for their efforts.

SELECTION OF THE BLOOD DONOR

Careful selection of donors is essential in order to prevent transmission of disease to recipients, and in order to protect the donor's state of health. It is neither practicable nor adequate to conduct a complete physical examination of all donors for the purpose of insuring freedom from transmissible diseases or to protect the donor against harm from donation of 500 ml. of blood. Certain routine questions regarding medical history and certain routine objective procedures are feasible, however, with every donor. These should be carried out in all instances in order to determine the ability of the donor to give blood suitable for transfusion. It is imperative that the donor's name, age, sex, weight, address, duty station, and telephone number be recorded for the purpose of future identification and location. The selection and processing of the donor normally can be satisfactorily accomplished by the blood bank technician. It is essential, however, that the operation of any blood donor service be supervised by a qualified physician.

If circumstances permit, a member of the blood bank staff should interview the prospective donor privately in an office. The interviewer must be patient and explain all terms in simple language, when necessary, in order that DD Form 572 may be completed accurately. The items which must be considered during this interview are discussed below.

Age

The usual permissible age range for blood donors is 21 to 60 years. Donors less than 21 years of age may be accepted if (1) they are members of the Armed Forces over 17 years of age, (2) they are civilian minors for whom signed consent is given by the parent or legal guardian, and (3) they are married and over 17 years of age. In practice, the state requirements concerning legal age of consent should be followed.

Previous Donation

A period of not less than 8 weeks should have elapsed since 500 ml. of blood was withdrawn from the individual. No more than five donations are allowable within a 12-month period. Exceptions may be made for individuals who weigh over 170 pounds, and in cases of polycythemia vera and uncomplicated essential hypertension. In these cases, the donor must present written permission from his physician and otherwise meet the requirements stated here.

Nourishment

Blood to be employed for transfusion purposes must be collected from donors who have refrained from eating fatty foods for at least 6 hours. This would include foods such as cream, butter, oleomargarine, soup, mayonnaise, fat meats, and cheese. It is preferable to permit donors to eat nonfatty meals rather than enforce a period of starvation. Too long a period of strict fasting lowers the blood sugar and increases the donor reaction rate. A suitable diet can include—(1) Bread or toast without butter, (2) coffee or tea without cream or milk, (3) jam or jelly, (4) sugar, and (5) fruit or fruit juices.

Medical History

The prospective donor should understand and supply proper information concerning the following specific conditions:

Malaria. Individuals who indicate a history of nonrecurrent malaria and who have not demonstrated any clinical symptoms of this disease for at least 2 years may be accepted. Persons who have lived in malarious areas and who have taken malarial suppressive drugs may be accepted under the following conditions:

1. They must not have received such medication during the preceding 2 years.
2. They must have been free of symptoms of malaria for at least 2 years since taking drugs.

The medical officer supervising the operation of the blood donor center may exercise discretion in accepting persons who have received light seasonal courses of drugs in the southern United States but who have never had clinical infection. Individuals with a history of malaria may be accepted as donors if the blood is to be used in the preparation of plasma or plasma fractions.

Syphilis. All prospective donors who indicate a definite history of syphilis are disqualified unless they can provide sufficient evidence that they have been treated adequately and are serologically nonreactive. If blood is collected from individuals who have had syphilis, have been adquately treated, and are presently serologically nonreactive, this information must be clearly recorded. Such blood must not be issued from the blood bank unless stored for over 96 hours in the refrigerator. If possible, this blood should be transfused only to serologically reactive recipients since the passive transfer or reagin may lead to confusion in the serological status of the recipient.

Jaundice. A history of viral hepatitis (infectious or homologous type) at any time in the past, automatically excludes a donor, except when blood is to be used for plasma fractions. This is a standard of the National Institutes of Health (Division of Biologic Standards), and should be strictly adhered to. All donors with a questionable history of jaundice, not clearly obstructive in nature, should be referred to a qualified physician for a decision to accept or reject the donor. Prospective donors whose intimate associates may be suffering from acute hepatitis should be rejected.

Undulant Fever (Brucellosis). Perspective donors with a history of undulant fever may be accepted if they have had no attack in the preceding 2 years.

Tuberculosis. All prospective donors having had active clinical tuberculosis are disqualified.

Upper Respiratory Disease. Persons having colds, fever, grippe (flu), persistent cough, sore throat, pain in the chest, or other indications of upper respiratory disease must be rejected until at least 1 week after active symptoms have subsided.

Skin Diseases. Individuals who have active local infections of the skin or of the mucous membranes, including diseases such as lupus erythematosus, should be disqualified. Prospective donors with uncomplicated fungal infection of the skin may be accepted except if the infection occurs on the arm from which blood is to be drawn. Following a tattoo, donors must not be accepted until 6 months have elapsed.

Allergic States. Persons indicating a history of repeated attacks of asthma, even if inactive at the time of examination, are disqualified. Allergies involving the gastrointestinal tract (diarrhea, etc.) are also cause for rejection. All other active allergic manifestations such as hay fever, hives, skin rash (including poison ivy) are cause for rejection. History of allergies of the latter type, but no active symptoms, does not disqualify the individual.

Hematologic Disorders. Any persons giving a history of polycythemia, excessive bleeding, or a blood dyscrasia should be referred to a physician or rejected.

Convulsions. Persons giving a history of convulsive seizures and frequent fainting (except in infancy) are not acceptable.

Heart Disease. Individuals who are known to have heart disease, such as rheumatic fever or coronary artery disease, are not acceptable. Nonacceptability also applies to persons who have hypertension with a systolic pressure above 200, diastolic above 100, or hypotension with a systolic pressure below 100 and a diastolic pressure below 50. Also, persons giving a history of shortness of breath, swelling of the feet and ankles, or angina or pain in the chest must be rejected.

Diabetes. Individuals requiring insulin for the treatment of diabetes are disqualified.

Drug Addiction. Persons who are drug addicts or who have a history of drug addiction should not be accepted as blood donors.

Inoculations and Vaccinations. Individuals who have been immunized against typhoid, typhus, plague, tetanus, cholera, poliomyelitis, influenza, and Rocky Mountain spotted fever are acceptable without qualification. A 2-week interval is recommended following any yellow fever inoculation. A 2-week period is also recommended following an immune reaction to smallpox vaccine. Donors treated for rabies are acceptable 1 year after the last treatment. Prospective donors who have received other injections should be referred to a qualified physician.

Alcoholism. Individuals under the influence of alcohol at the time of examination are not acceptable.

Surgery. Persons who have undergone major surgical operations are not accepted until at least 3 months following the surgery.

Dental Extraction. Extraction of a tooth within a period of less than 1 week is cause for rejection.

Transfusions. Individuals who have been transfused with human blood or its derivatives within the preceding 6 months are not acceptable as blood donors.

Gastrointestinal Disorders. Persons under active treatment for ulcers, or those with any other active gastrointestinal disorders should not be accepted.

Weight Loss. A history of recent and unexplained weight loss of 10 pounds or more should disqualify an individual as a blood donor. Exception is made for individuals on diets intended to reduce excessive weight.

Cancer. Individuals with an established history of cancer should be referred to the physician in charge of the blood bank.

Pregnancy. Pregnant women and those who have delivered within 3 months are not acceptable as blood donors.

Kidney Disease. Individuals who give a history of any active infections such as nephritis will be rejected. All others should be screened by a qualified physician.

Personality Disorders. Persons manifesting outward signs of neurotic or psychotic tendencies should be rejected. This type of situation requires great tact and a qualified physician should be consulted.

Illness in Last Month. Particular note should be made of the presence or repeated occurrence of upper respiratory infections. No arbitrary limit should be set regarding the interval after which the donor becomes eligible for donating blood because of the variability, severity, and duration of such infections. Donors with chronic sinusitis and hay fever are acceptable if they are not in the acute stage and are otherwise in good health. Detailed information should be obtained from donors who give a history of septic sore throat within the preceding 3 months, and they should be accepted only after consultation with the physician.

Presence of Leuko-Agglutinins. Agglutinins have been discovered in the plasma of certain individuals which agglutinate leukocytes and thrombocytes. These leuko-agglutinins are present in increased titer in such conditions as anemias, idopathic thrombocytopenic purpura, collagen diseases, and leukemias. Among these individuals possessing leuko-agglutinins and experiencing transfusion reactions there have been a striking correlation between the severity of the reaction and the agglutinin titer. The blood bank technician, then should consider that under ordinary circumstances a history of conditions such as those indicated above with the possible associated presence of such leuko-agglutinins would preclude the use of an individual as a blood donor.

Note. Additional considerations are included on the DD Form 572. Other illnesses or abnormalities may be added as causes for rejection as experience indicates. In cases of doubt, the physician should be consulted.

Physical Examination of the Donor

Following the interview relevant to medical history, the donor must be given an abbreviated physical examination in order to determine his weight, temperature, blood pressure, pulse rate, and hemoglobin concentration. The criteria for these measurements are indicated below.

Weight. Donors who weigh more than 130 pounds may give a full 500 ml. of blood along with pilot samples. Donors whose weight falls between 110–130 pounds should not have over 300 ml. of blood withdrawn. Donors weighing less than 110 pounds should not be accepted.

Temperature. An oral temperature above 99.6° F. disqualifies the donor. The temperature must be recorded with a suitable thermometer held in the closed mouth for at least 2 minutes. Thermometers should be cleansed with surgical green soap and soaked in 70 percent ethyl alcohol for a minimum of 10 minutes between each usage.

Blood Pressure. Blood pressure should be determined with a properly functioning sphygmomanometer, the cuff of which is placed around the upper arm of the individual. Blood pressure must be greater than 100/60 and less than 200/100. Unusual blood pressures and irregularities of the pulse should be referred to a qualified physician for interpretation.

Pulse Rate. The pulse rate of an acceptable donor should be between 60–100 per minute.

Hemoglobin. The acceptable donor hemoglobin level is 12.5 gm./100 ml. of blood for females and 13.5 gm./100 ml. of blood for males. The technique for determining hemoglobin concentration is discussed below.

Hemoglobin Determination

It is of great importance to determine whether or not a prospective donor has a sufficient amount of hemoglobin to donate blood and still be able to supply his bodily needs. The most practical and commonly used method for measuring hemoglobin concentration is the copper sulfate procedure. A solution of copper sulfate with a specific gravity of 1.053 for female donors or 1.055 for male donors at 25° C. is recommended. These values correspond to 12.5 gm. and 13.5 gm. of hemoglobin, respectively.

The method is based on the fact that whole blood dropped into a solution of copper sulfate of known specific gravity is encased in a sac of copper proteinate, and that the specific gravity of this isolated drop is not changed for about 15 to 20 seconds. The rise or fall of the drop during this interval indicates whether it is lighter or heavier than the solution. The rise (or suspension) or fall of the single drop of blood in this solution within the first 15 to 20 seconds will indicate whether the donor is acceptable or should be rejected. If the drop rises or remains suspended, the donor is not acceptable. If the drop of blood sinks, the donor is acceptable (at least on the basis of hemoglobin level).

APPARATUS:

Beaker, 600 ml.

Capillary tubes. (A fresh capillary tube must be used for each determination.)

Rubber bulbs. (These rubber bulbs must fit the ends of capillary tubes and may be used repeatedly.)

Individual sterilized lancets. (These lancets must be sterile to prevent the transmission of the hepatitis virus.)

REAGENT:

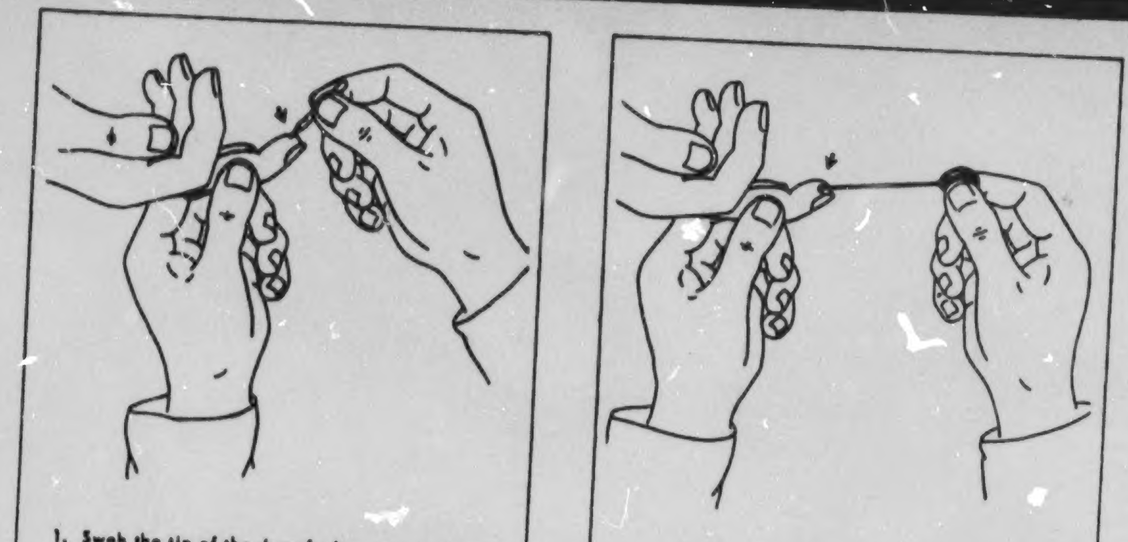
Copper sulfate solution. (Directions for preparing this reagent are included in app I.)

Care of the Reagent

The copper sulfate solution, when not in use, should be tightly stoppered to prevent evaporation and changes in specific gravity. When the environmental temperature is high, it may be necessary to immerse the copper sulfate container in an ice water bath and stopper it between each determination. One determination can be made per millimeter of copper sulfate solution. Best results, however, are obtained if not more than 75 percent of the possible determinations are made in any given volume of solution. At least 1 minute should elapse between each determination to allow the solution to clear. The surface of the solution should be kept clear and free from fragments. Convection currents which might cause false readings should be avoided. When cold bottles of solutions are brought into a warm room, they should be allowed to reach room temperature before being used.

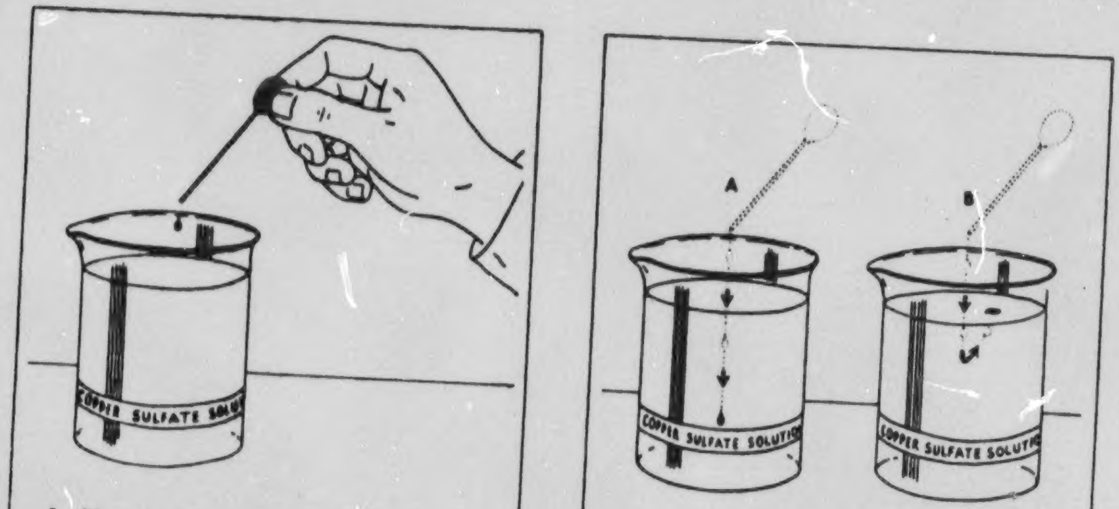
TECHNIQUE:

1. When the donor reports to the technician, his DD Form 572 should be checked for completeness and used for positive identification.
2. The finger selected for puncture should be carefully cleansed with alcohol. If necessary, the finger should be scrubbed with a soapy sponge before applying the alcohol.
3. The puncture should be made on the side of the tip of the finger with sufficient force so that an adequate amount of blood is secured without milking the finger.
4. After wiping off the first drop with a sterile dry sponge, sufficient blood should be



1. Swab the tip of the donor's finger with alcohol, and puncture the skin with a sterile lancet.

2. Place the tip of the capillary tube into the drop of blood. Blood will enter the tube.


COPPER SULFATE SOLUTION
A
B
COPPER SULFATE SOLUTION
COPPER SULFATE SOLUTION

3. Place the index finger over the hole in the bulb, and deliver one drop of blood to the copper sulfate solution.

4. The blood drops a short distance in the solution. If the blood drop continues to fall the donor is acceptable (4a). If the drop rises to the top, the donor is not acceptable (4b).

Figure 10-2. Copper sulfate method for determining hemoglobin concentration.

collected in the capillary tube so that there will be a slight excess after a full drop has been expelled.

5. The delivery end of the capillary tube should be held about one-half inch above a 4-inch column of the solution. The tube may be supported on the side of the beaker. Gentle steady pressure should be applied to the bulb and one drop delivered into the center of the solution surface.

INTERPRETATION:

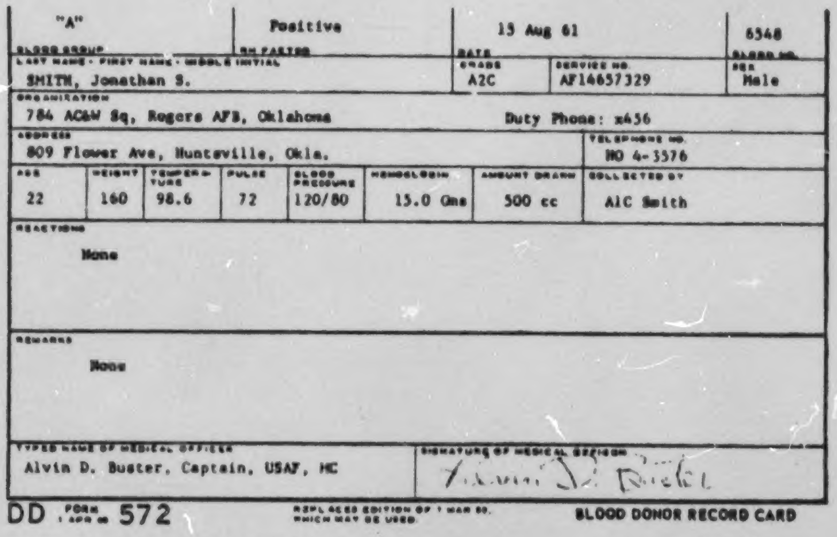
1. The person is considered acceptable if the drop of blood is heavier than the test solution and continues to sink during the first 15 seconds after it has entered the solution.
2. The person is also considered acceptable if the drop is of the same specific gravity as the test solution and becomes stationary during the 15 seconds following its entrance into the solution.
3. The individual is considered ineligible to donate if the drop is lighter than the test solution and rises, perhaps only a few millimeters, during the 15 seconds before starting to sink to the bottom. The test should be repeated if the action of the drop is questionable or unsatisfactory. In the case of doubt after retesting, it is better to reject a donor who might possibly be made anemic if accepted with a borderline hemoglobin level. Any prospective donor who does not have the required concentration of hemoglobin should be referred to the physician in charge of the blood bank. This technique is illustrated in figure 10-2.

Completion of Forms

All of the preceding information concerning the donor is indicated on DD Form 572 (figs. 10-3 and 10-4).

When a donor has been screened and has been determined to be acceptable for the donation of 500 ml. of blood, he is assigned a blood unit serial number. This number should be typed or stamped in the specified area on DD Form 572. At this time the donor number should also be stamped on the collecting container label and the labels of two sterile pilot tubes which should be securely attached to the container. In addition, the donor number should be recorded in waterproof ink. This tag is attached to the blood collecting container. The donor is then ready to donate blood and is sent to the donation room with his stamped DD Form 572, blood collecting container, and pilot tubes.

A log book should be maintained in the blood donor center listing the donor's name and assigned serial number, serial number of donor bottle, blood group, Rh type, and date drawn.



"A" BLOOD GROUP	Positive RH FACTOR	15 Aug 61 DATE	6548 BLOOD NO.

LAST NAME - FIRST NAME - MIDDLE INITIAL SMITH, Jonathan S.	GRADE A2C	SERVICE NO. AF14657329	SEX Male

ORGANIZATION
784 AC&W Sq, Rogers AFB, Oklahoma Duty Phone: x456

ADDRESS 809 Flower Ave, Huntsville, Okla.	TELEPHONE NO. HO 4-3576

AGE	WEIGHT	TEMPERATURE	PULSE	BLOOD PRESSURE	HEMOGLOBIN	AMOUNT DRAWN	COLLECTED BY
22	160	98.6	72	120/80	15.0 Gms	500 cc	AIC Smith

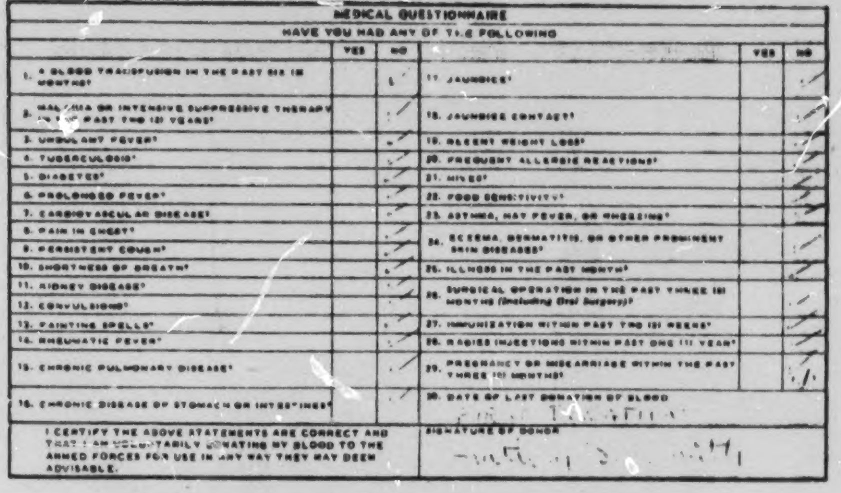
REACTIONS
None

REMARKS
None

TYPED NAME OF MEDICAL OFFICER Alvin D. Buster, Captain, USAF, MC	SIGNATURE OF MEDICAL OFFICER

DD FORM 572 REPLACES EDITION OF 1 MAR 50, WHICH MAY BE USED. BLOOD DONOR RECORD CARD

Figure 3. DD Form 572 "Blood Donor Record Card".



MEDICAL QUESTIONNAIRE

HAVE YOU HAD ANY OF THE FOLLOWING

	YES	NO		YES	NO
1. A BLOOD TRANSFUSION IN THE PAST SIX (6) MONTHS?		✓	17. JAUNDICE?		✓
2. MALARIA OR INTENSIVE SUPPRESSIVE THERAPY IN THE PAST TWO (2) YEARS?		✓	18. JAUNDICE CONTACT?		✓
3. UNDULANT FEVER?		✓	19. RECENT WEIGHT LOSS?		✓
4. TUBERCULOSIS?		✓	20. FREQUENT ALLERGIC REACTIONS?		✓
5. DIABETES?		✓	21. HIVES?		✓
6. PROLONGED FEVER?		✓	22. FOOD SENSITIVITY?		✓
7. CARDIOVASCULAR DISEASE?		✓	23. ASTHMA, HAY FEVER, OR WHEEZING?		✓
8. PAIN IN CHEST?		✓	24. ECZEMA, DERMATITIS, OR OTHER PROMINENT SKIN DISEASES?		✓
9. PERSISTENT COUGH?		✓			
10. SHORTNESS OF BREATH?		✓	25. ILLNESS IN THE PAST MONTH?		✓
11. KIDNEY DISEASE?		✓	26. SURGICAL OPERATION IN THE PAST THREE (3) MONTHS (Including Oral Surgery)?		✓
12. CONVULSIONS?		✓			
13. FAINTING SPELLS?		✓	27. IMMUNIZATION WITHIN PAST TWO (2) WEEKS?		✓
14. RHEUMATIC FEVER?		✓	28. RABIES INJECTIONS WITHIN PAST ONE (1) YEAR?		✓
15. CHRONIC PULMONARY DISEASE?		✓	29. PREGNANCY OR MISCARRIAGE WITHIN THE PAST THREE (3) MONTHS?		✓
16. CHRONIC DISEASE OF STOMACH OR INTESTINES?		✓	30. DATE OF LAST DONATION OF BLOOD [illegible]		
I CERTIFY THE ABOVE STATEMENTS ARE CORRECT AND THAT I AM VOLUNTARILY DONATING MY BLOOD TO THE ARMED FORCES FOR USE IN ANY WAY THEY MAY DEEM ADVISABLE.			SIGNATURE OF DONOR		

Figure 10-4. Medical questionnaire on reverse side of DD Form 572.

COLLECTION OF BLOOD USING PLASTIC CONTAINERS

Standard blood collecting equipment consists of a plastic container with an integral donor tube. A plastic blood pack is illustrated in figure 10-5. The stepwise method for using the blood pack is discussed below.

1. Cans containing plastic blood bags should be opened with a rotary type can opener to avoid damaging the plastic with the blade of the opener or the sharp edge of the opened can. If glass pilot tubes are used, two should be attached to the bag prior to filling. The appropriate donor number will be stamped on the bag and pilot tubes. At each donor table, means of suspending the plastic pack should be prepared. The plastic pack should be hung by one of the holes in the top seal. The support should be arranged so that the plastic bag is as far below the donor's arm as possible without pulling on the needle or kinking the donor tubing (fig. 10-6).
2. Apply a blood pressure cuff. Place the cuff with the tubes pointed toward the shoulder with the lower edge of the cuff well above the intended site of venipuncture. Inflate the cuff to 60 mm. of mercury to assist in locating a vein. Release the pressure in the cuff before preparing the skin.
3. Prepare the skin area.
   - *a.* Using a cotton swab or sponge, tincture of green soap is applied over an area 4 x 4 inches. The skin is rubbed vigorously with the tincture of green soap so that foam is produced, since the mere application of the soap will not have the desired cleansing effect.
   - *b.* The soap and dissolved dirt and fat are removed with a solution of 70 percent alcohol (either ethyl or isoprophyl).
   - *c.* After cleansing, the skin is treated with tincture of iodine (3.5 percent) by applying at the center and working outward (fig.10-7).
   - *d.* If the donor is not allergic to procaine, give one-fourth ml. of a 1 percent solution intradermally approximately one-fourth inch distal to the actual site where the needle will enter the vein. Use procaine syrettes when available. If these are not available, use a separate sterile 26 gage needle for each donor (fig. 10-8).
   - *e.* Skin sensitivity to iodine may occur. Consequently, the iodine must be removed after it has dried

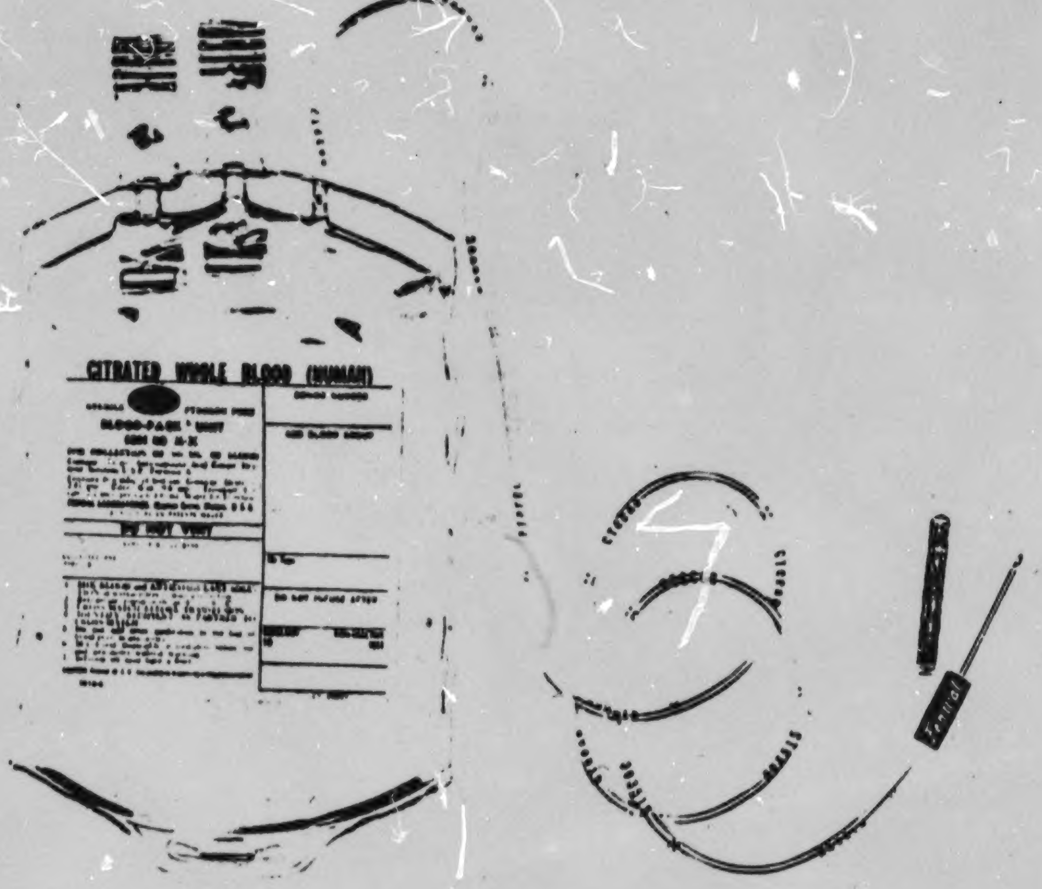


Figure 10–8. Plastic blood pack.

Seventy percent alcohol is used for this purpose.

f. The area is now prepared for immediate phlebotomy and the venipuncture may be performed. If a necessary delay occurs before venipuncture can be accomplished, the prepared area should be protected by the application of sterile sponges wet with 70 percent alcohol (fig. 10–9).

Note. Preparation of the donor's arm should always be conducted from the venipuncture site outward. The cleansed area should never be touched after preparation of the venipuncture site. Contamination of the needle and other portions of equipment must be avoided inasmuch as undetected contamination of blood has, on occasion, resulted in death of transfused patients.

4. Make a loose overhand knot in the integral donor tubing of the blood pack, 3 or 4 inches from the needle.
5. Suspend the blood pack on the previously prepared support.
6. Again inflate the pressure cuff to 60 mm. of mercury and maintain this pressure throughout the phlebotomy.
7. Grasp the needle hub firmly, twist the cover to break the seal, then pull off (fig. 10–10).

480

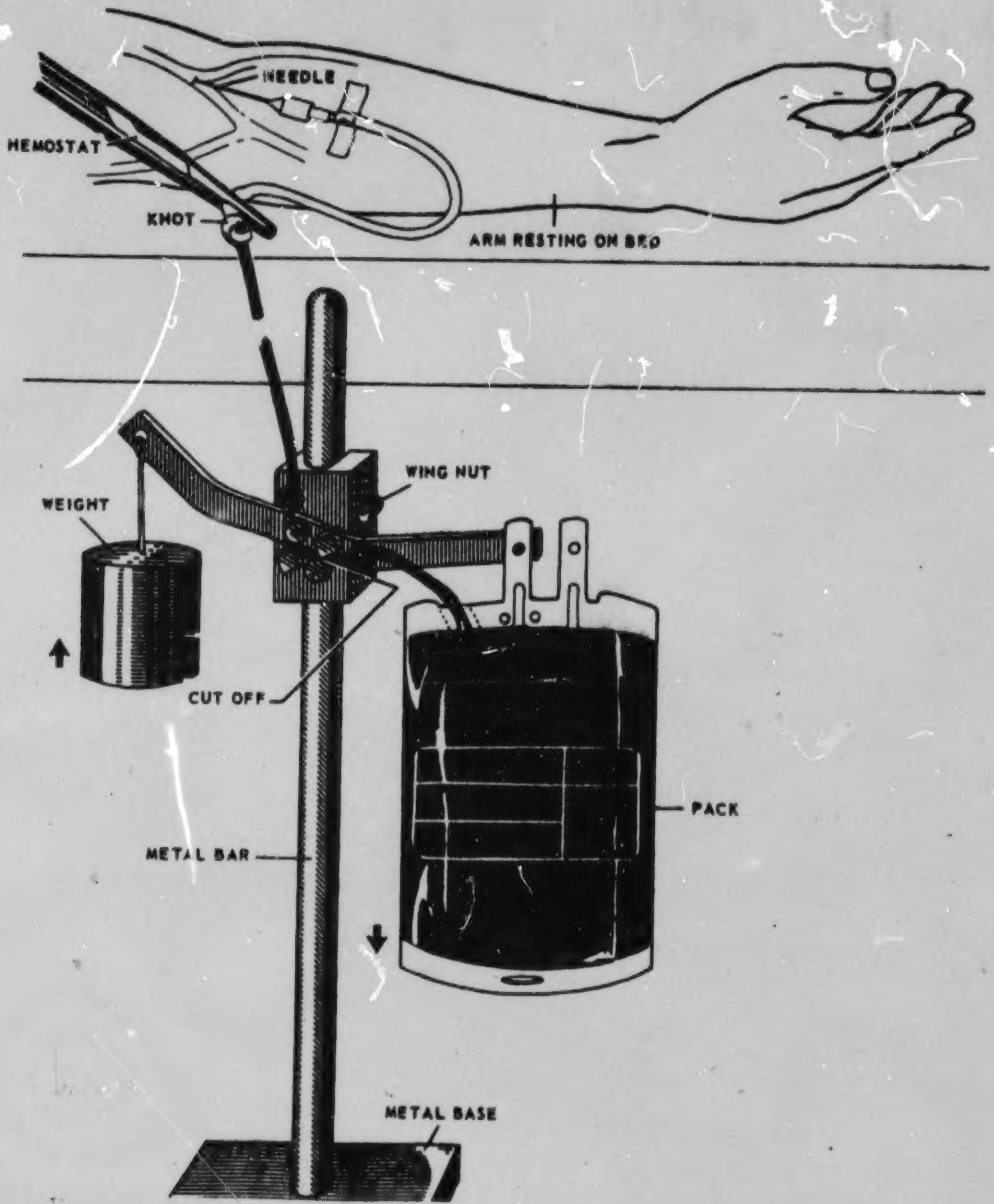

NEEDLE
HEMOSTAT
KNOT
ARM RESTING ON BED
WING NUT
WEIGHT
CUT OFF
PACK
METAL BAR
METAL BASE

Figure 10-6. Automatic shut-off blood collecting balance. Apparatus rests at a level lower than the patient's arm. Adjustable height is controlled by wing nut attached to sliding metal rectangle which moves vertically up and down on the metal bar.

494

481

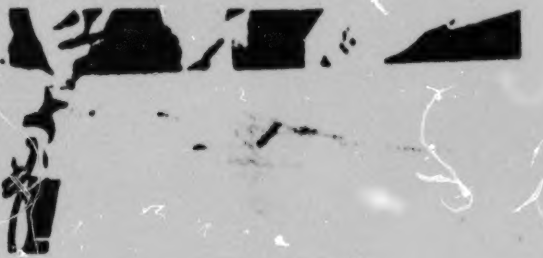


Figure 10-7. Prepare the skin area with green soap, followed by alcohol and tincture of iodine. Apply at the center and work outward.

Figure 10-8. If the donor is not allergic to procaine, inject ¼ ml. intradermally approximately ¼ inch distal to the site of venipuncture.

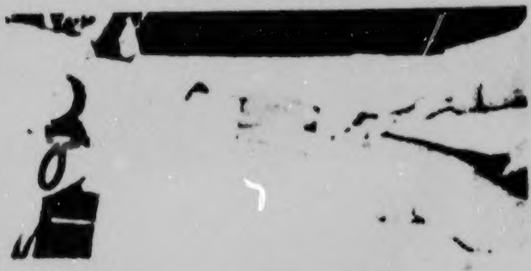


Figure 10-9. The area is now prepared for immediate phlebotomy and the venipuncture may be performed.

Figure 10-10. After removing the needle cover perform the venipuncture.

Figure 10-11. To release the bead at the base of the donor tube, squeeze the tubing above the bead and move the bead completely free into the blood pack.

8. Perform the venipuncture. Insert the needle, with the beveled side up, well into the vein. (The needle can be secured in position with adhesive tape.)
9. Release the bead in the base of the donor tube to permit blood flow. Squeeze the tubing above the bead between thumb and forefinger to move the bead completely free into the blood pack (fig. 10-11).
10. Mix the blood and anticoagulant. Mixing should begin as soon as the blood flow has been established. Gently elevate the bottom of the blood pack several times at six or seven intervals during collection to insure thorough mixing. Avoid tension on the tubing that might disturb the needle in the vein.
11. Fill the plastic bag so that it is just barely firm but not taut (fig. 10-12).

12. Release the pressure in the cuff. Retain the pressure cuff in position for possible use when taking the pilot tube samples.
13. Avoid disturbing the needle when tightening the knot. A drop of alcohol on the knot aids in setting it (fig. 10-13).

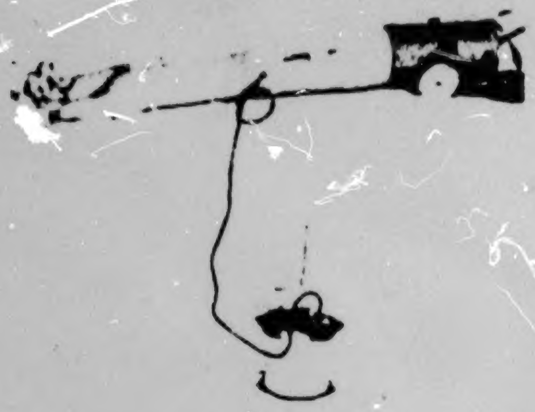


Figure 10-12. Fill the plastic bag so that it is just barely firm but not taut.

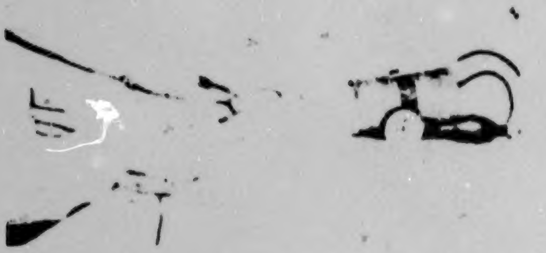


Figure 10-13. Tighten the knot in the donor tubing. A drop of alcohol aids in setting it.

14. Pinch the tubing tightly immediately above the knot. Apply a hemostat to the tubing about 1 inch above the pinched area. Release the pinch and cut the tubing at the pinch site.
15. The two pilot tubes for serology, typing and crossmatching may be collected from the cut end of the donor tube by releasing the hemostat (fig. 10-14). Pressure may be reapplied in the pressure cuff if necessary to obtain the desired amount of blood for samples.

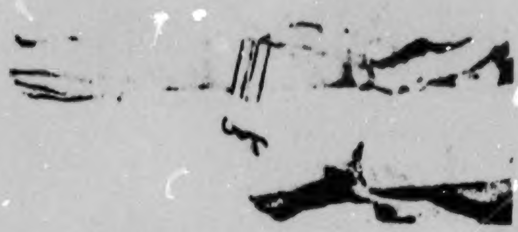
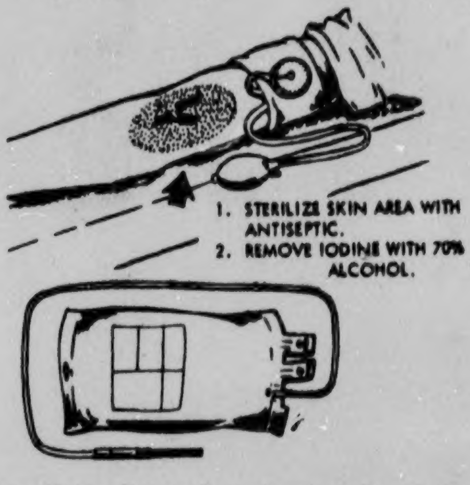


Figure 10-14. The pilot tube specimens are collected from the cut end of the donor tube by releasing the hemostat.

16. *Be sure the tourniquet pressure is released.*
17. Exert gentle pressure with a sterile sponge immediately above the site of venipuncture and withdraw the needle. Elevate the donor's arm vertically without flexion at the elbow, and have him apply firm pressure on the sponge to prevent blood leakage from the vein and consequent hematoma formation. This phlebotomy technique is summarized in figures 10-15 through 10-19.



1. STERILIZE SKIN AREA WITH ANTISEPTIC.
2. REMOVE IODINE WITH 70% ALCOHOL.

Figure 10-15. Prepare the venipuncture site with tincture of green soap, alcohol, and tincture of iodine. Remove the excess iodine with 70 percent alcohol.

18. Apply a sterile dressing over the site of puncture.
19. Allow the donor to rest 10-15 minutes.
20. Turn the blood pack end for end at least 10 times.
21. Store the filled blood packs in the refrigerator at 5°±1° C. Blood should be refrigerated promptly to assure maximum preservation of the red cells.

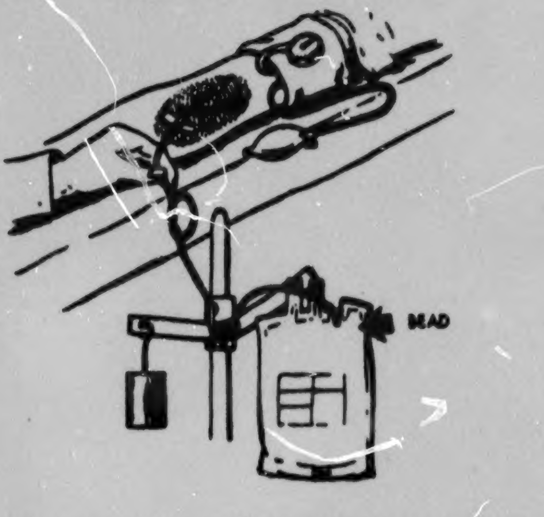


Figure 10-16. Mount the blood pack on the automatic shut-off blood collecting balance. Make a loose knot in the donor tubing and perform the venipuncture.

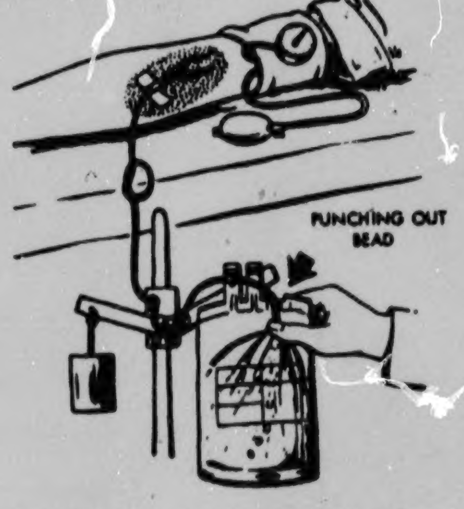


Figure 10-17. After the insertion of the needle in the vein, squeeze the bead from the base of the donor tube into the blood pack.

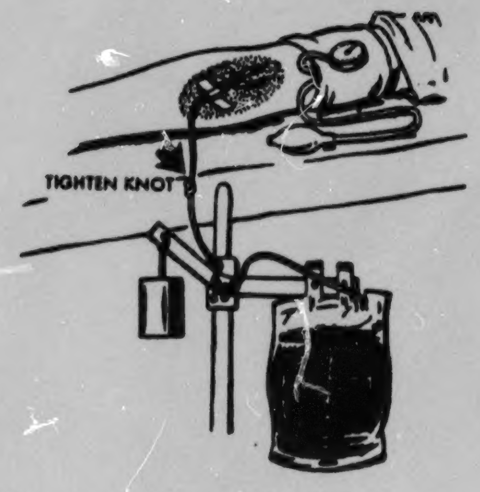


Figure 10-18. Allow the blood pack to fill until it is firm and then tighten the knot in the donor tube.

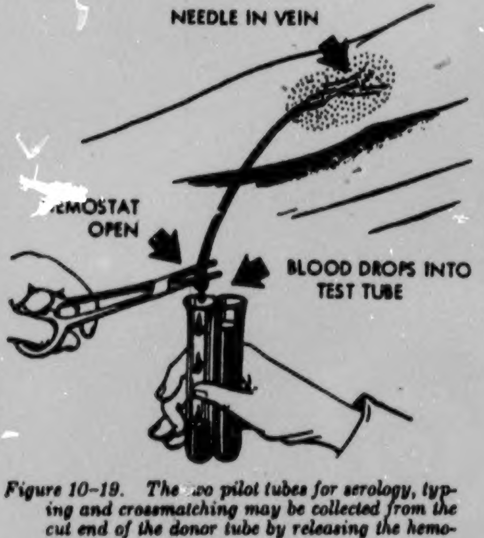


Figure 10-19. The two pilot tubes for serology, typing and crossmatching may be collected from the cut end of the donor tube by releasing the hemostat.

22. An alternate procedure for collecting pilot tubes after the bag has filled with blood is to first place a clamp on the donor tubing where it enters the bag. Then remove the needle from the vein and insert it into a vacuum blood collecting tube that has been attached to the bag prior to collecting the blood. After the blood has been drawn out of the donor tubing, set a knot near the needle hub. Release the clamp and fill the donor tubing with ACD blood from the plastic bag. The donor tubing may now be tied in segments of seales with a dielectric sealer. The blood collected in the tube will be attached to the bag prior to storage at 5°±1° C. (fig. 10-21.)

23. A second alternate procedure for collecting pilot tubes is to first place a clamp on the donor tubing where it enters the bag. Then withdraw the needle from the donor's arm leaving the tubing filled with

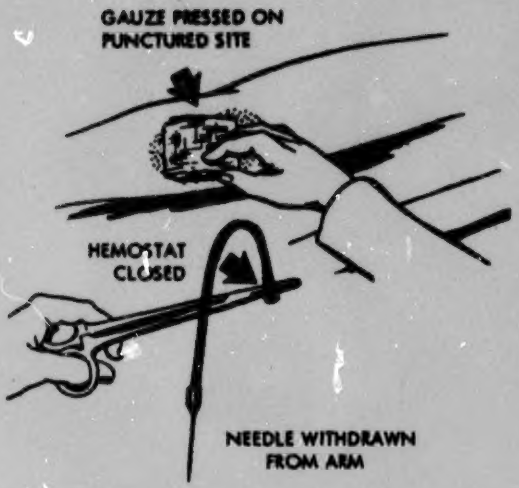


Figure 10-20. Release the tourniquet pressure. Exert gentle pressure with a sterile sponge immediately above the site of venipuncture and withdraw the needle.

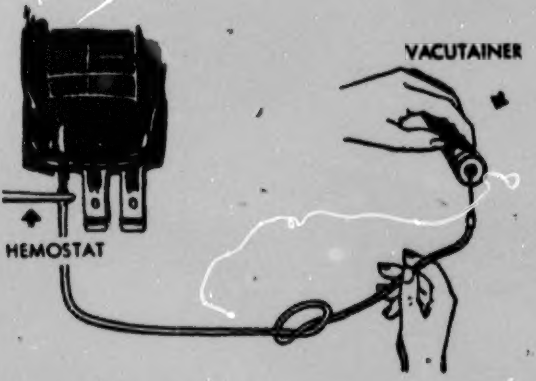


Figure 10-21. An alternate procedure for collecting pilot tube specimens.

blood. The bag and tubing are then taken to the dielectric sealer and seals are made at the places indicated by the crosses, working from the bag toward the needle. The displacement by the seals expresses from the needle about 2 ml. of blood which may be collected in a dry tube for serology. It is important to permit this blood to be freshly expressed or pressure will build up in the plastic tubing, preventing proper sealing. It is also important to carry out the sealing operation within an hour of blood collection or clot retraction may give rise to uneven distribution of cells and serum in each segment. The tube remains attached to the bag and the needle is cutoff close to the final seal. For storage in the refrigerator, the segmented tube is folded into a neat bundle by bending at the seals and an elastic band is slipped around it to keep it in place, (fig. 10-22).

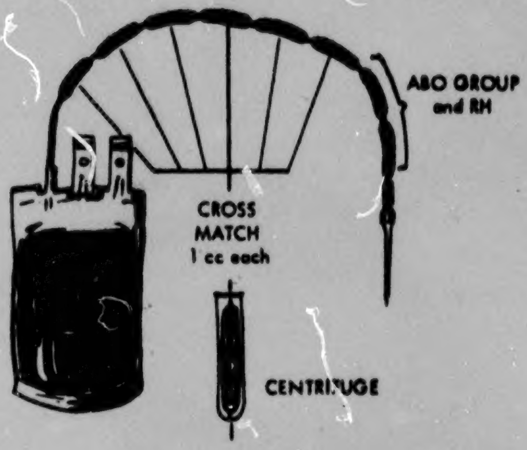


Figure 10-22. A second alternate procedure for collecting pilot tube specimens.

24. To obtain an aliquot of blood for culture, mix the blood thoroughly; then strip the blood from the donor tube into the container. Prevent backflow into the tubing while thoroughly mixing the contents again. Allow the blood to reflux into the donor tube. Prepare a segment of the desired length by knotting or dielectric sealing. This procedure assures a representative sample of the contents of the container with no risk of contamination of the residual contents.

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BLOOD DONOR REACTIONS

Although the withdrawal of blood is usually accomplished with no untoward reaction, a small percentage (4–6 percent) of donors exhibit some systemic reaction before, during, or following the venipuncture. Occasionally, there may be local injury at the site of venipuncture, syncope, infection, tetany, or systemic reactions such as reactions due to procaine sensitivity. The first two are probably the most common accidents.

Local injury at the site of venipuncture most commonly occurs from puncturing the wall of a vein opposite the site of entry, thus producing a hematoma. Several measures may be taken to prevent hematomas. They are (1) proper entry of the needle into the vein, (2) correct positioning of the arm prior to venipuncture, and (3) use of a sharp needle. The most common error in technique is that of partially withdrawing the needle after the vein wall has been punctured. Every effort should be made to prevent hematomas since they produce skin discoloration and cause pain. Experienced personnel usually have little difficulty.

Venipuncture produces a portal for possible entry of bacteria into subcutaneous tissue and occasionally a localized infection results, producing a cellulitis or even an abscess of the arm. They may be prevented by proper sterilization of equipment, proper cleansing and preparation of the area, and avoidance of contamination of this area prior to venipuncture.

Syncope (fainting) is a common donor reaction. There may be many predisposing factors, such as fear, hunger, pain, drawing an excessive amount of blood, lack of rest, an overheated room, low blood pressure, and excessive rapidity of withdrawing blood. Syncope may, to some extent, be prevented by proper screening of donors, reassuring those who are apprehensive, serving fruit juice prior to donation, avoiding overdrawing, and collecting blood in a cool room. The treatment of syncope consists of stopping the blood collection, inhalations of aromatic spirits of ammonia, and keeping the donor supine while elevating his feet. A cold, wet towel placed over the forehead is also of value. The donor should never be left alone.

Occasionally convulsions may occur. A mouth gag should be placed between the jaws immediately to prevent biting the tongue. The donor should be held to prevent his falling from the donor table and then removed to a bed for at least 20–30 minutes.

Serious cardiovascular effects from blood donation are rare. An emergency tray should be maintained with the following stimulants: Epinephrine, 1:1,000, caffeine sodium benzoate, and coramine. An oxygen tank and mask should be kept in the collection room. In cases of any emergency, a physician should be called at once.

Tetany is seldom encountered. The donor, however, may, on rare occasions, suffer from the loss of calcium. The symptoms and signs include muscular spasms of the extremities, especially at the wrists, rapid breathing, and apprehension. Inhalations of carbon dioxide and use of intravenous calcium gluconate are indicated. A physician should be notified immediately.

Note. A report of each donor reaction must be recorded on the respective DD Form 572.

A medical kit for the treatment of severe reactions and accidents should be on hand during all blood collecting operations. It is suggested that items of equipment and supplies listed below be available at all times in the recovery room.

1. Unsterile:
    - *a.* Recovery beds or cots.
    - *b.* Blankets.
    - *c.* Shock blocks.
    - *d.* Emesis basin.
    - *e.* Bedpan and urinal.
    - *f.* Small cylinder of oxygen with mask and gage.
    - *g.* Mouthgags.
    - *h.* Armboard.
    - *i.* Tourniquet.
    - *j.* Paper towels.
    - *k.* Mouth wipes.
    - *l.* Handtowels.
    - *m.* Adhesive tape.
    - *n.* Paper bags.
2. Sterile basic suture set:
    - *a.* Small scissors (1).
    - *b.* Mouse-tooth forceps (1).
    - *c.* Small needle holder (1).
    - *d.* Dermal or silk sutures with a traumatic cutting edge needle.
    - *e.* Plain catgut.

   f. 2 x 2 sponges.
   g. 2-inch bandages.
   h. Fluted towels for drapes (2).
   i. Recipient set (1).
   j. Syringes with needles, 2 ml.
   k. Syringes with needles, 20 ml.

3. Drugs:
   a. Ampoules of—
      (1) Aromatic ammonia.
      (2) Caffeine sodium benzoate.
      (3) Aminophylline.
      (4) Epinephrine (1:1,000).
      (5) Coramine.
      (6) Ephedrine.
      (7) Calcium gluconate (10 ml.).
      (8) Sodium amytal.
      (9) Atropine.
      (10) Distilled water.
      (11) Magnesium sulfate, 25 percent (10 ml.).
   b. Elixir of phenobarbital.
   c. Aspirin.
   d. Glucose, 5 percent in saline.
   e. Glucose, 5 percent in water.
   f. Dextran (2 units).

LABORATORY PROCESSING OF DONOR BLOOD

When donor blood specimens are received in the laboratory for potential use in blood transfusion, the following must be accurately determined.

1. ABO Group.
2. Rh Type.
3. Presence of atypical antibodies.
4. Serologic test for syphilis.

The various tests required to make these determination are customarily run as a battery for the sake of simplicity and accuracy. The procedures employed must conform to good and acceptable techniques and meet National Institutes of Health (Division of Biologic Standards), standards.

The ABO grouping must be ascertained using potent anti-A and anti-B and anti-AB (group O) sera and confirmed using known, fresh group A and B cells.

The Rh typing for Rh_o (D) must be determined using potent anti-Rh_o (D) typing serum. (For specimens found to be Rh_o (D) negative, additional testing is required.)

The presence of atypical antibodies in the donor's serum is determined using the indirect Coombs techniques with pooled, fresh group O cells. (For specimens demonstrating evidence of atypical antibodies, additional testing is required.)

APPARATUS:

Test tubes: Kahn or culture tubes 10 x 75 mm.
Test tube rack.
Medicine droppers (dropettes, Pasteur dropper, or pipettes).
Centrifuge.

REAGENTS:

Anti-A grouping serum (NIH approved).
Anti-B grouping serum (NIH approved).
Anti-Rh_o grouping serum (NIH approved).
Anti-rh″ grouping serum (NIH approved).
Anti-hr′ grouping serum (NIH approved).
Anti-hr″ grouping serum (NIH approved).
Known O, A, and B cells (NIH approved).
Saline.

ABO BLOOD GROUPING

For determination of the ABO blood group, at least two separate tests should be done, using the following procedures:

1. Determination of the cell group by testing the blood with known sera using either the slide method or the test tube method outlined below; or
2. Confirmation of or proving the blood group by determining the serum group against known A and B cells.

Slide Method of Cell Grouping

1. Mark a glass micro slide "A" on the left and "B" on the right, and identify with the number or name of the blood sample being tested. Divide the slide by marking through the center with a wax pencil.
2. Place one drop of anti-A serum in the square marked "A" and one drop of anti-B serum in the square marked "B."

3. With the aid of a wooden applicator or pipette, transfer a small amount of the blood specimen (not greater than one-half the volume of serum used) to each serum. One drop of a 2 percent saline suspension of cells may be used. Blood from a finger stick, blood containing citrate or oxalate, or clotted blood may be used.
4. Mix well and observe for agglutination.
5. When tests are made with potent anti-serum agglutination will occur within a few seconds except in the case of weak A or A,B cells. Agglutination of A_1, A_2, and A_1B and A_2B cells will be delayed, but with potent sera good agglutination in most cases will be observed within 1 minute and in all cases within 3 minutes.

Test Tube Method of Cell Grouping

1. Mark one tube "A" and a second "B." Write the number or name of the blood being tested on each tube.
2. Place one drop of anti-A serum in tube A and 1 drop of anti-B in tube B.
3. Make a 2 percent suspension in saline of the blood specimen and add one drop to each tube.
4. Shake well and centrifuge for 1 to 2 minutes at 1,000–2,000 r.p.m.
5. Gently dislodge button of cells and read for agglutination. In a rare instance, doubtful results may be checked by viewing the test tube with the scanning lens of the microscope or by placing a drop of the mixture on a slide and viewing it with the low power objective.

The group is determined on the basis of agglutination or absence of agglutination of anti-A and anti-B sera as indicated below.

Blood Group	*Anti-A Serum*	*Anti-B Serum*
AB	+	+
A	+	−
B	−	+
O	−	−

+ = Agglutination
− = No Agglutination

Do not apply heat; the action of these iso-agglutinins is not enhanced and may be weakened or reversed by heat (particularly with weakly reacting cells).

The known Group A and B cells will be drawn in Alsever's solution or EDTA each week, and two sets of test cells will be washed and suspended daily. One set will be used by the transfusion service and the other will be used for the serology and processing section.

Note. Procedure should be modified according to instructions with each lot of antisera.

ANTI-A AND ANTI-B TITERS IN GROUP O SERUM

Current standards governing the use of low-titer group O blood as universal donor dictate that anti-A and anti-B agglutination titers greater than 1:50 shall be excluded. To establish this level of antibody titers, the following technique is employed:

TECHNIQUE:

1. Prepare four tubes labeled as follows:
   (1) Immune Anti-A.
   (2) Immune Anti-B.
   (3) Natural Anti-A.
   (4) Natural Anti-B.
2. To tubes 1 and 2 add two drops of the donor's serum and two drops of a 3 percent suspension of fresh cells; in tube 1, A_1 cells and in tube 2, B cells. These two tubes should then be incubated at 37° C. for 1 hour and examined for hemolysis.
3. To tubes 3 and 4, add two drops of a 1:50 dilution of donor serum in saline. To tube 3 add two drops of 3 percent suspension of fresh A_1 cells and to tube 4 add two drops of 3 percent suspension of fresh B cells.
4. Spin at 1,000 r.p.m., 1 minute and examine for evidence of agglutination.

INTERPRETATION:

The presence of hemolysis in tube 1 or 2 indicates the presence of immune antibodies and the blood should not be used as a universal donor. The presence of agglutination in tube 3 or 4 indicates a titer of 1:50 or greater of natural-occurring antibodies and this blood should not be used as a universal donor.

Note. Procedure should be modified according to instructions with each lot of antisera.

DIRECTIONS FOR USE OF ANTI-A, B (GROUP O) SERUM BLOOD GROUPING SERUM (HUMAN)

Blood of groups A_1, A_2, B, A_1B, and A_2B react with anti-A,B serum. Group O blood fails to react.

Tube Method of Testing

1. Prepare a 2 percent suspension in normal saline or normal group compatible serum of cells from freshly drawn, clotted, oxalated, or citrated blood.
2. Add one drop of anti-A,B (group O) serum to a test tube (10 x 75-mm.).
3. Add two drops of the 2 percent cell suspension.
4. Centrifuge 1 minute at 500 to 1,000 r.p.m.
5. If desired, readings may be confirmed with the aid of a small hand lens (3x-3.5x-6x) or a magnifying mirror.

Note. Procedure should be modified according to instructions with each lot of antisera.

CONFIRMATION OF OR PROVING THE BLOOD GROUP BY AGGLUTININ TESTING

The blood group is ordinarily obtained by testing the red cells with known anti-A and anti-B sera. To assure accuracy, the blood grouping is confirmed by testing the serum with known group A_1 and group B red blood cells for the serum's content of anti-A and anti-B agglutinins. This test is also known as serum grouping, reverse grouping, or back grouping.

Testing the serum with known group A_1 and B red cells suspensions in a test tube is sensitive and rapid, and should be employed routinely.

1. Mark one test tube "A" and a second tube "B" and write patient's name or code number of the blood on each tube.
2. Place one to three drops of the serum to be tested in each tube.
3. Add one drop of a freshly prepared, 2 percent saline suspension of known group A_1 or a pool of at least five random group A cells to the tube marked "A," and one drop of a freshly prepared, 2 percent saline suspension of group B cells to the tube marked "B."
4. Shake well and centrifuge for 1 to 2 minutes at 1,000 to 2,000 r.p.m.
5. In reading, avoid hard shaking as this may break up agglutination.

Blood Group	*Antibody Present in Serum*
AB	Neither
A	Anti-B
B	Anti-A
O	Anti-A and Anti-B

6. Hemolysis due to the presence of active complement in fresh serum may partially or completely obscure agglutination. Hemolysis turns the saline pink or light red; enough cells may remain to be seen with the naked eye. Hemolysis may be prevented by inactivating serum for 10 minutes in a 56° C. water bath.

Note. Agglutination due to iso-agglutinins anti-A and anti-B is not enhanced at 37° C. and warming may actually weaken or reverse agglutination. The application of heat either to the slides on which blood grouping is done or to tube tests should be avoided. Anti-A and anti-B react best at 4° C. Therefore, when a problem is encountered, the tubes for both the forward and reverse typings should be placed at 4° C. with the patient's own cells and serum as a negative control for cold agglutinins.

SUBGROUPING OF GROUP A AND AB BLOODS

Human red cells of group A and AB may show marked variation in the speed and intensity of agglutination with anti-A agglutinin. This has led to the recognition of various subgroups of A and AB. Cells which are agglutinated rapidly and completely with anti-A antibody are designated A_1 or A_1B. Cells which react less rapidly and less completely with the same serum are designated A_2 or A_2B, etc. A special serum which has been adsorbed so that it will react only with red cells of subgroups A_1 or A_1B (not with other cells known to be of subgroups A_2 or A_2B) is required to determine the A subgroups. The serum is called adsorbed anti-A serum. Cells of group A which react with this serum are of blood subgroups A_1 or A_1B. Group A red cells which fail to be agglutinated by this serum are usually called A_2 or A_2B, etc. A reagent that reacts similarly to this serum may be extracted from certain seeds, e.g., from seed of dolichos biflorus.

The qualitative or quantitative differences between red cells of blood group A are cause for only occasional concern in blood transfusion. Cases have been reported in which individuals of blood group A_1 had antibodies for cells of blood groups A_2 and O. Individuals of blood group A_2 or A_2B may have antibodies for cells of blood group A_1. These possibilities must be considered in the crossmatching of blood and in the study of reactions to blood transfusions. However, the transfusion of group A blood without regard to its subgroups is considered safe practice when coupled with adequate crossmatching.

Reactions of Subgroups of A

Group	*Reagents used for Identification*	*Results*
A_1	Anti-A	Strongly positive.
	Anti-A_1 lectin	Strongly positive.
	Anti-H lectin	Negative.
A_{1-2} (A^{int})	Anti-A	Strongly positive.
	Anti-A_1 lectin	Weakly positive.
	Anti-H lectin	Weakly positive.
A_2	Anti-A	Strongly positive.
	Anti-A_1 lectin	Negative.
	Anti-H lectin	Strongly positive.
A_3	Anti-A	Weakly positive; appearance of mixed agglutination with many free cells left unagglutinated.
	Anti-A_1 lectin	Negative.
	Anti-H lectin	Strongly positive.

Rh TYPING

Clumping of specific red cells by the Rh slide testing antibody is dependent upon a high concentration of both protein and red cells. For the test, therefore, the typing sera must have a protein concentration of 6 percent or more (preferably 25 to 30 percent) and the cells must be suspended in concentrations of approximately 40 to 50 percent in plasma or serum. Oxalated or heparinized blood is most satisfactory for testing. If the blood is clotted, cells may be obtained from the clot and suspended in a heavy concentration in the serum surrounding the clot. For the test, two parts of blood are mixed with one part of serum.

Cells suspended in saline are unsatisfactory and should never be used in slide tests.

For optimal reactions, the surface of the viewing box must be 45° to 50° C.

Slide Test (With Slide Test Serum)

1. Place two drops (each equal in volume to the drop of serum) of the blood to be tested on a micro slide.
2. Add one drop of anti-Rh_0 (anti-D) slide test serum to the two drops of blood.
3. Mix and spread over most of the slide.
4. Place directly on the glass plate of a viewing box containing a light globe which will bring the temperature of the plate to 45° to 50° C.
5. Rotate back and forth, observing for agglutination.
6. The test is completed within 2 minutes. Do *not* read after two minutes.
7. Clumping indicates that the cells are Rh_0, (D) positive; no agglutination indicates the cells are Rh_0, (D) negative.

Modified or Rapid Test Tube Method

1. In a small tube place one drop of slide test anti-Rh_0 (anti-D) serum.
2. Using freshly collected whole blood, prepare approximately 2 percent suspension of cells in their own serum or plasma, in A,B serum, or in 20 percent bovine albumin solution. It is also recommended that the small amount of cells which clings to a wooden applicator after it has been swirled in the whole blood sample, may be transferred directly to the tube containing the test serum. Using this technique it is extremely important to make sure that no pieces of fibrin are carried over into the test serum.
3. Transfer a drop of 2 percent cell suspension, or that amount of cells which cling to an applicator, to the tube containing the serum and mix well.

4. The tube should be thoroughly shaken and centrifuged at 1,000 to 2,000 r.p.m. for 2 minutes. The addition of two to three drops of saline at this point, facilitates reading the test and does not interfere with agglutination.
5. Gently disengage the cell sediment from the bottom of the tube, exercising care not to break up agglutination.
6. Agglutination of the cells indicates that they are Rh_o (D) positive; no agglutination indicates the cells are Rh_o (D) negative.

Sources of Error in Rh Typing

1. Weak or nonstandardized anti-Rh typing sera.
2. Bacterial contamination of serum.
3. Chemical and biochemical deterioration.
4. Improper storage of anti-Rh typing serum. (Keep in the refrigerator when not in use.)
5. Misunderstanding of the specificity of the serum employed.
6. Misunderstanding of the variety of antibody in the serum employed.
7. Improper blood specimen.
8. Improper cell suspensions.
9. Temperature during testing.
10. Excessive agitation of test tube mixtures after centrifugation.
11. Doubtful or weak slide reactions.
12. Failure to use positive and negative controls.
13. Rh typing in the neonatal period.
14. Error in identification.
15. A positive Direct Coombs test may produce false positive reactions.

Note. Procedure should be modified according to instructions with each lot of antisera.

DIRECTIONS FOR THE USE OF ANTI-Rh TYPING SERUM (HUMAN) ANTI-Rh_o (ANTI-D)

Stick Method of Testing

1. Add one drop of anti-Rh_o (anti-D) slide test serum to a small test tube (10 x 75 mm.).
2. With one or two applicator sticks, transfer sufficient cells from the blood sample (clotted, oxalated, citrated, or heparinized blood may be used) to constitute a 2 percent suspension and centrifuge for 1 minute at 1,000 r.p.m. (If desired, test may be incubated at 37° C. for 15 minutes before centrifugation.).
3. Add one drop of saline to facilitate reading.
4. Resuspend the packed cells by gentle agitation and examine for agglutination.

Note. Procedure should be modified according to instructions with each lot of antisera.

Saline Tube Method of Testing

1. Prepare a 2 percent suspension in normal saline of washed cells from clotted, oxalated, citrated, or freshly drawn blood.
2. Add one drop of anti-Rh_o (anti-D) saline tube test serum to a small test tube (10 x 75 mm.).
3. Add two drops of the 2 percent cell suspension.
4. Incubate in a 37° C. water bath for 60 minutes.
5. Examine sedimented cells for agglutination.
6. If no agglutination is visible, shake the tube and centrifuge for 1 minute at 1,000–2,000 r.p.m.
7. Resuspend the packed cells by gentle agitation and examine for agglutination.
8. If desired, a reading may be confirmed with the aid of a small hand lens (3x–3.5x–6x) or a magnifying mirror.

THE SALINE TUBE METHOD FOR Rh_o (D) IS NOT USED FOR ROUTINE Rh_o TESTING. THE METHOD USED FOR ROUTINE Rh_o (D) TESTING IS THE STICK METHOD, USING ANTI-Rh_o (ANTI-D) SLIDE TEST SERUM.

Note. Procedure should be modified according to instructions with each lot of antisera.

DIRECTIONS FOR THE USE OF ANTI-rh′ (ANTI-C) BLOOD TYPING SERUM

Modified Tube Method of Testing

1. Prepare a 2 percent suspension of cells in their own or normal group, compatible serum.
2. Add one drop anti-rh′ (anti-C) slide test serum to a small test tube (10 x 75 mm.).
3. Add two drops of the 2 percent cell suspension.
4. Incubate in a 37° C. water bath for 15 minutes.
5. Centrifuge for 1 minute at 500 to 1,000 r.p.m.
6. Resuspend the packed cells by gentle agitation.
7. If desired, readings may be confirmed with the aid of a small hand lens (3x–3.5x–6x) or a magnifying mirror.

DIRECTIONS FOR THE USE OF ANTI-rh″ (ANTI-E) BLOOD TYPING SERUM

Modified Tube Method of Testing

1. Prepare a 2 percent suspension of cells in their own or normal group compatible serum.
2. Add one drop of anti-rh″ (anti-E) slide test serum to a small test tube (10 x 75 mm.).
3. Add two drops of the 2 percent cell suspension.
4. Incubate in a 37° C. water bath for 15 minutes.
5. Centrifuge for 1 minute at 500 to 1,000 r.p.m.
6. Resuspend the packed cells by gentle agitation.
7. If desired, readings may be confirmed with the aid of a small hand lens (3x–3.5x–6x) or a magnifying mirror.

Note. Procedures should be modified according to instructions with each lot of antisera.

DIRECTIONS FOR THE USE OF ANTI-hr′ (ANTI-c) BLOOD TYPING SERUM

Serum for slide test and modified tube test is prepared from pools of albumin agglutinins and is designed for use either on the slide or in the test tube. In contrast to saline agglutinins, albumin agglutinins cannot be used with cells suspended in saline.

Tube Method

1. Prepare a 2 percent suspension of cells in their own or normal group compatible serum.
2. Add one drop anti-hr′ (anti-c) slide test serum to a small test tube (10 x 75 mm.).
3. Add two drops of the 2 percent cell suspension.
4. Incubate in a 37° C. water bath for 30 minutes.
5. Centrifuge for 1 minute at 500 to 1,000 r.p.m.
6. Resuspend the packed cells by gentle agitation and examine for agglutination.
7. If desired, readings may be confirmed with the aid of a small hand lens or a magnifying mirror.

Note. Procedure should be modified according to instructions with each lot of antisera.

DIRECTIONS FOR THE USE OF ANTI-hr″ (ANTI-e) BLOOD TYPING SERUM

Anti-hr″ (anti-e) serum is useful in studying those rare cases in which transfusion or pregnancy has resulted in immunization against this blood factor. It is also of value in the proper selection of a panel of test cells for determining the specificity of an unknown antibody.

Saline Tube Test

1. Prepare a washed 2 percent suspension in normal saline of the cells to be tested.
2. Add one drop of the anti-hr″ (anti-e) serum to a small test tube (10 x 75 mm.).

3. Add two drops of the 2 percent cell suspension.
4. Incubate in a 37° C. water bath for 60 minutes.
5. Examine sedimented cells for agglutination.
6. If no agglutination is visible, shake the tube and centrifuge for 1 minute at 1,000 to 2,000 r.p.m.
7. Resuspend the packed cells by gentle agitation and examine for agglutination.
8. If desired, readings may be confirmed with the aid of a small hand lens of a magnifying mirror.

Note. Procedure should be modified according to instructions with each lot of antisera.

ROUTINE PROCEDURE FOR DETECTION OF D^u BLOODS

D^u may be considered as a modified D antigen and should be regarded as equally antigenic with the normal D factor. Therefore, bloods screened as Rh negative by any method of testing should be further tested for the D^u factor by the following procedure.

TECHNIQUE:

1. Prepare a 2 percent suspension in normal saline of the blood in question.
2. Place two drops of the suspension into each of two small test tubes.
3. Add one drop of anti-Rh_0, (anti-D) slide test serum to one of the test tubes. Mix well.

   Note. Anti-D slide test serum used with antihuman serum for detection of D^u must be completely specific for the D factor.
4. Add one drop of 22 percent Bovine Albumin to the second test tube which serves as a control. Mix well.
5. Incubate both tubes at 37° C. for 15 minutes.
6. Remove from incubator and wash cells in each tube three or four times with tubes full of fresh saline. Decant completely after the last washing.
7. Add two drops of antihuman serum to each tube. Shake well. Centrifuge for 1 minute at 1,000 r.p.m.
8. Resuspend the packed cells by gently agitating and examine for agglutination.

Note. Procedure should be modified according to instructions with each lot of antisera.

INTERPRETATION:

If there is no agglutination in either tube, the blood can be classified as Rh negative.

When agglutination appears only in the tube which received the anti-Rh_0 (anti-D) slide test serum, the blood is of the D^u variety and should be classified as Rh positive.

If the contents of both tubes are agglutinated, this will indicate that the cells were nonspecifically coated and cannot be accurately tested for the D^u factor. If from a donor, these bloods should be classified as Rh positive. Patients or recipients who present such doubtful results should be classified as Rh negative.

DIRECTIONS FOR USE OF ANTI-rh^w (ANTI-C^w) BLOOD TYPING SERUM

Anti-rh^w (anti-C^w) serum is useful in studying those cases in which transfusion or pregnancy has resulted in immunization against this blood factor. It is also of value in the proper selection of a battery of test cells for determining the specificity of an unknown antibody.

Method of Testing

Note. This serum contains incomplete antibodies reacting only with serum-suspended red cells in the test tube.

1. Prepare a 2 percent suspension in their own serum (or normal A.B serum) of the cells to be tested.
2. Add one drop of anti-rh^w (anti-C^w) serum to a small test tube (10 x 75 mm.).
3. Add two drops of the 2 percent cell suspension.
4. Shake well and incubate at 37° C. for 1 hour.
5. Centrifuge at 1,000 r.p.m. for 1 minute.
6. Gently resuspend the sediment and examine macroscopically for agglutination.

Cells that contain the anti-rh^w (anti-C^w) factor will be agglutinated. The rh^w (C^w) negative cells will resuspend smoothly.

Note. Procedure should be modified according to instructions with each lot of antisera.

Cardiolipin Microflocculation Test

All donor blood must be checked serologically using an appropriate test for syphilis—STS. Any blood exhibiting a reactive or weakly reactive result must not be used for transfusion. If a reactive or weakly reactive individual is encountered, the information should be reported to the appropriate physician who will handle the delicate problem involved.

SELECTION OF GROUP O BLOOD FOR UNIVERSAL DONOR USE BY BLOOD BANKS

In certain emergencies, time will not permit the transfusion of group specific blood. In these cases, transfusion of the individual, irrespective of blood group, with the safest blood available is desired. Certain group O bloods contain isohemagglutinins of sufficient titer so that when infused into a recipient of blood groups A, B, or AB, they may cause hemolysis of the recipient's cells. Serious reactions are uncommon, however, and in life or death emergencies when the proper blood is not available, any group O blood can be used. Selected group O bloods should be kept on hand in anticipation of emergencies requiring transfusion without normal crossmatching procedure. The tests indicated in table 10–1 should be performed on these bloods. The procedure for selection of group O bloods for disaster situations differs from table 10–1 and is discussed in chapter 12.

Table 10–1. Tests for Selection of Group O Universal Donor Blood for Use by Blood Bank

Test	Required results in order for blood to be satisfactory for use as universal donor.
ABO grouping	Group O.
Proof grouping of donor's serum with known A and B cells.	Donor serum should agglutinate both A and B cells.
D (Rh_o) typing	D (Rh_o) negative.
C (rh'), E (rh''), and D^u typing if negative for D.	C (rh'), E (rh''), and D^u negative.
Natural anti-A and anti-B hemagglutinin titer.	Natural anti-A and anti-B agglutinin titer less than 1:50.
Immune anti-A and anti-B agglutinin titer.	No agglutination or hemolysis in the presence of complement.
Indirect Coombs for antibody screen.	Negative.

Note. Units of blood fulfilling these criteria should be so marked and refrigerated in an easily accessible location.

Recording of Information

All data obtained from the preceding tests must be accurately and completely recorded on the blood container label, DD Form 572, and Donor Ledger (discussed later). Each blood container label is completed according to the ABO group, Rh type, serologic reaction, and expiration date. The labeling as to group, type, unit numbers, and pilot tube numbers should also be rechecked at this time. The final blood container label should appear as illustrated in figure 10–23① and 10–23②.

Previous Records and Repeat Testing

A donor's previous record of ABO grouping and Rh typing should not be accepted for identification of units of blood subsequently collected from the same donor. New determinations should be made for each collection. The identification of the blood, i.e., ABO grouping and Rh typing, should be repeated on the pilot tube sample whenever the agency which performs the crossmatch procedure is not the same as the collecting agency.

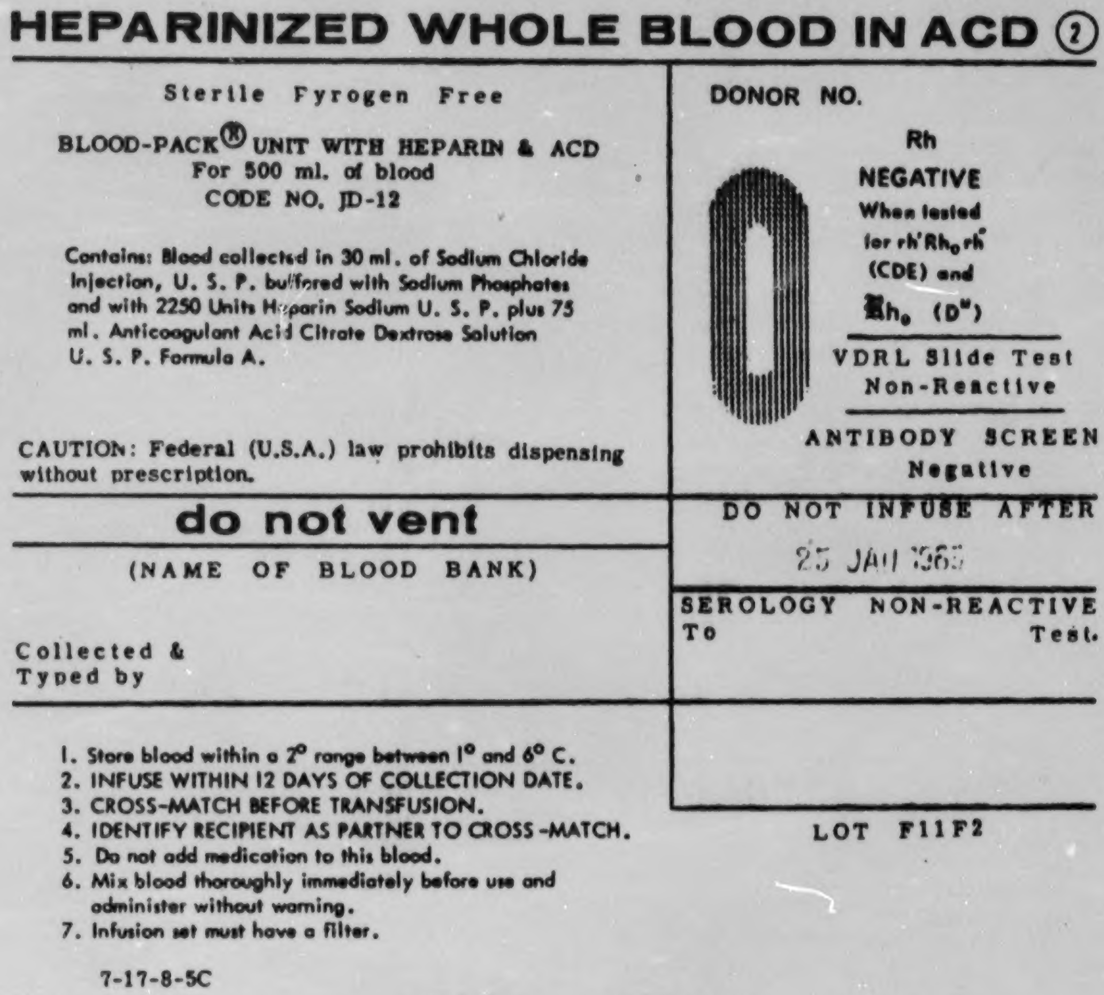
Note. All blood samples should be stored at 1°–6° C. for at least 4 days after transfusion to permit a laboratory reexamination, if necessary.

STORAGE AND EXCHANGE OF BLOOD

Once donor blood is collected and processed, it must be properly stored. In addition, transportation and exchange of blood between hospitals are occasionally required. Information dealing with each of these considerations is presented below.

Storage

Blood must be stored at a temperature range of 1° C. to 6° C. Immediately after the blood is collected, it should be placed in a refrigerator and its temperature brought to the optimal level as



HEPARINIZED WHOLE BLOOD IN ACD ②

Sterile Pyrogen Free

BLOOD-PACK® UNIT WITH HEPARIN & ACD
For 500 ml. of blood
CODE NO. JD-12

Contains: Blood collected in 30 ml. of Sodium Chloride Injection, U. S. P. buffered with Sodium Phosphates and with 2250 Units Heparin Sodium U. S. P. plus 75 ml. Anticoagulant Acid Citrate Dextrose Solution U. S. P. Formula A.

CAUTION: Federal (U.S.A.) law prohibits dispensing without prescription.

do not vent

(NAME OF BLOOD BANK)

Collected &
Typed by

DONOR NO.

O

Rh
NEGATIVE
When tested
for $rh'Rh_0rh''$
(CDE) and
Rh_0 (D^u)

VDRL Slide Test
Non-Reactive

ANTIBODY SCREEN
Negative

DO NOT INFUSE AFTER

25 JAN 1965

SEROLOGY NON-REACTIVE
To Test.

1. Store blood within a 2° range between 1° and 6° C.
2. INFUSE WITHIN 12 DAYS OF COLLECTION DATE.
3. CROSS-MATCH BEFORE TRANSFUSION.
4. IDENTIFY RECIPIENT AS PARTNER TO CROSS-MATCH.
5. Do not add medication to this blood.
6. Mix blood thoroughly immediately before use and administer without warming.
7. Infusion set must have a filter.

LOT F11F2

7-17-8-5C

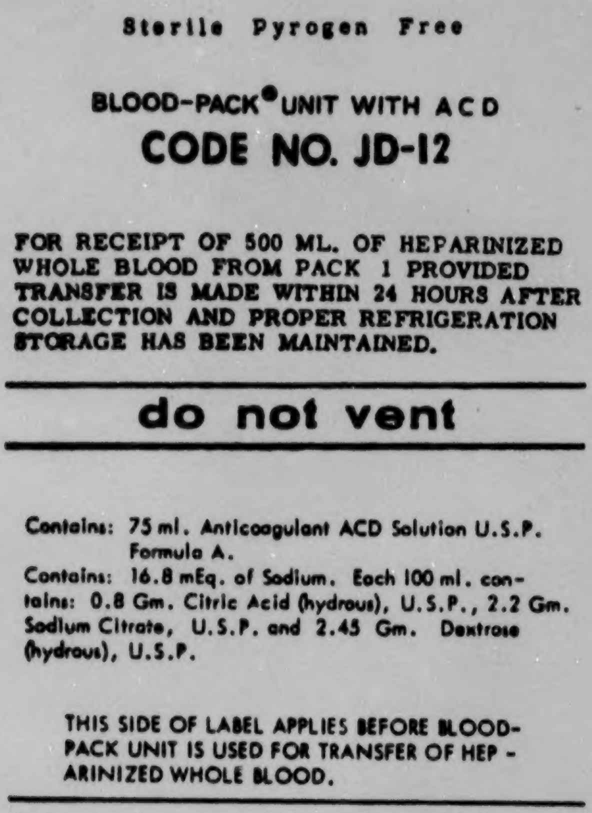
Figure 10-23①. Blood container label.

quickly as possible. A uniform temperature with a variation of plus-1 or minus-2 degrees within the range of 1° C. to 6° C. is of the utmost importance to obtain the longest survival of the red cells.

If the temperature remains constant, whole blood collected in ACD anti-coagulant solution will maintain satisfactory transfusion properties for 21 days after its collection from the donor. Blood collected in sodium citrate for the purpose of preparing plasma, should not be used after a 5-day period following blood collection. Concentrated red cell preparations from which the plasma has been removed should, however, be used within 3 hours. Stored blood should be checked daily for any evidence of hemolysis or an excessively cloudy supernatant plasma (which may indicate bacterial contamination).

Blood should be stored in a refrigerator that is coupled to a temperature recording device and provided with an alarm system which is activated if the proper temperature is not maintained. If possible, the motor should be detached from the body of the refrigerator, since the vibrations of a motor will increase any tendency toward occurrence of hemolysis. The blood containers should be placed in the refrigerator at least one-half inch apart in order to maintain an even temperature throughout. A typical blood bank refrigerator is illustrated in figure 10-24.

Note. Blood should not be stored near dry ice.



Sterile Pyrogen Free

BLOOD-PACK® UNIT WITH ACD
CODE NO. JD-12

FOR RECEIPT OF 500 ML. OF HEPARINIZED WHOLE BLOOD FROM PACK 1 PROVIDED TRANSFER IS MADE WITHIN 24 HOURS AFTER COLLECTION AND PROPER REFRIGERATION STORAGE HAS BEEN MAINTAINED.

do not vent

Contains: 75 ml. Anticoagulant ACD Solution U.S.P. Formula A.
Contains: 16.8 mEq. of Sodium. Each 100 ml. contains: 0.8 Gm. Citric Acid (hydrous), U.S.P., 2.2 Gm. Sodium Citrate, U.S.P. and 2.45 Gm. Dextrose (hydrous), U.S.P.

THIS SIDE OF LABEL APPLIES BEFORE BLOOD-PACK UNIT IS USED FOR TRANSFER OF HEPARINIZED WHOLE BLOOD.

*Figure 10-23*①—continued.

Storage of blood in refrigerators on wards and in operating rooms not directly under the supervision of blood bank personnel is not recommended. Any blood which has been removed from the blood bank, which has not been administered as planned to the patient, and which has not been uncapped or opened in any way, should be returned immediately to the blood bank. The blood bank should accept no responsibility for returned blood which has been out of its control.

Exchange

Every effort should be made to cooperate with blood banks of other organizations for the common purpose of supplying blood of proper group and type when needed. Trading or exchange of blood between banks is most often necessary in instances in which Rh negative blood of groups AB and B are involved. Since group O, Rh negative blood is valuable for universal donor purposes, it is often scarce and must be exchanged. Occasionally, other groups and types are involved in interbank exchange. Whenever it is necessary to ship blood from one bank to another, the following requirements should be met:

1. The blood bank making shipments of blood from one state to another, should be licensed by the National Institutes of Health (Division of Biologic Standards).
2. The blood being shipped should have been withdrawn from donors under the direct supervision of the blood bank which is shipping the blood.
3. The blood should have been withdrawn, in accordance with standard procedures, from donors who qualify according to the standard examination procedures.
4. The contents of the bottle should be free of clots, hemolysis, and other abnormali-

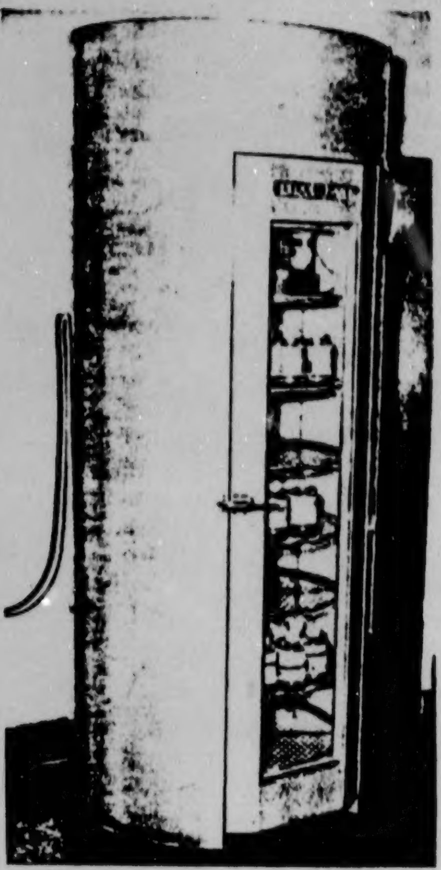


Figure 10-24. Blood bank refrigerator.

ties. As full a collection as is available should be sent.

5. The blood shipped should be the freshest blood available to the blood bank and, in any case, not over 7 days should have elapsed since withdrawal from the donor. Any deviation from this requirement should be acceptable to the blood bank making the request.
6. The bottle should be properly labeled, showing the donor's name, number, blood group, Rh type, result of serologic test, and date of withdrawal and expiration date. The label should also indicate the name and address of the blood bank responsible for the withdrawal of the blood.
7. The bottle of blood should be accompanied by an adequate sample of sterile clotted blood for the purpose of furnishing cells and serum for further tests at the blood bank requesting the shipment. This pilot tube should be firmly attached to the bottle.
8. Blood should be shipped in cartons which will provide temperatures below 10° C. for the length of time necessary to complete the shipment or for not less than 6 hours after packing.
9. The issuing blood bank should make every effort to use the fastest mode of transportation available in the shipment of blood.
10. The blood bank shipping the blood should notify the blood bank making the request of (*a*) the means by which blood is carried, (*b*) the approximate time of arrival, and (*c*) the route.

Disposition of Outdated Blood

Military blood banking operations will result in the accumulation of varying amounts of whole blood which is not used prior to its expiration date. The constituents of this outdated blood remain as a source of valuable biologic products. As a matter of policy, all appropriate and practicable means will be employed to insure optimum conservation and utilization of these constituents.

Feasible courses of action to implement this policy include the two listed below.

1. A plasma storage program maintained at the local blood bank consisting of single or double units of group specific plasma.
2. A plasma exchange program between individual blood banks and plasma fractionating laboratories.

TRANSPORTATION OF BLOOD

The transportation of blood and blood products is a natural outgrowth of blood banking. Blood must, on occasion, be transported between hospitals and from donor centers to hospitals, war zones, and disaster areas. The same pinciples are involved whether one unit is being shipped a short distance, or many units are being shipped for several hundred miles. Slight shaking of blood during transportation does not ordinarily produce sufficient trauma to the erythrocytes to result in significant amounts of hemolysis. The plasma probably acts as a partial buffer and, to some extent, protects the cells from injury. The effects of low atomospheric pressure must be considered in transportation. This problem is solved by maintenance of airtight, secure closure of bottles, which stabilizes the pressure within the container and prevents displacement of the cap. Important considerations pertinent to shipment of blood from donor centers to processing laboratories follow.

Preparation of Shipping Containers

1. The insulated chest-type shipping containers must be precooled to 4°–6° C. before loading with blood. This must be accomplished by one of the following methods:
   a. Place the entire shipping container, including racks and ice compartment in a walk-in refrigerator at 4°-6° C. for at least 12 hours. Be sure the lid is left open at least 1 inch
   b. Fill the ice compartment with chopped wet ice 12–24 hours before loading (do not open the shipping container during this precooling period).
2. The knockdown type cubicle does not require precooling. This is a prefabricated fiberboard box designed to contain 9 bottles or 18 bags of blood and equipped with 2 polyethylene sacks to contain wet ice (fig. 10–25).
3. All blood must be shipped as soon as possible after collection, using an approved blood shipping container which is sealed, prior to release to a shipping agent or carrier. It must be in the processing laboratory not later than midnight of the day after the blood is collected.

Procedure for Shipping Blood

1. Place the blood in the shipping container. (Insulated chests must be precooled before loading.)
2. Be sure the ice container is filled with fresh chopped wet ice.
3. Secure a blood inventory to the inside lid of each shipping container. Inventories will be listed on DD Form 573 (Shipping Inventory of Blood Collections) (fig. 10–26).
4. Wire the following information to the consignee for each shipment:
   a. Number of bottles or bags in shipment.
   b. Time of departure and flight or train number on which shipment was forwarded.
   c. Estimated time of arrival at destination.

Every effort should be made to utilize shipping containers to the maximum capacity. Each shipping container will be sealed before it is released to the shipping agent, and the seal will not be broken until the shipping container arrives at the designated laboratory, unless re-icing is required. Recipient sets must be included with bottles or bags.

After the prefabricated fiberboard box is loaded and the hinged cover is closed, the box should be sealed by means of three strips of rayon filament reinforced pressure-sensitive tape, 1-inch wide (or equivalent). The tape should extend from a point 3 inches in from the edge of the bottom to the corresponding point on the cover. A suitable tape is that standardized by the Air Force, tape, pressure sensitive adhesive paper backing; opaque; water resistant; 3 inches inside diameter of core, 60 yard roll, 1-inch wide, FSN 8135-290-0914.

Transportation Instructions

The officer in charge of the blood donor center will give the instructions enumerated below to the transportation officer.

1. All blood shipping containers will be packed with bottles or bags of blood, the can or polyethylene bag filled with chipped wet ice, and the box sealed before it is turned over to a carrier.
2. Blood will not be released to the carrier prior to 1 hour before plane, bus, or train departure.
3. If the scheduled train, bus, or plane departure is delayed after the blood has been released to the carrier, the transportation officer will be contacted by the

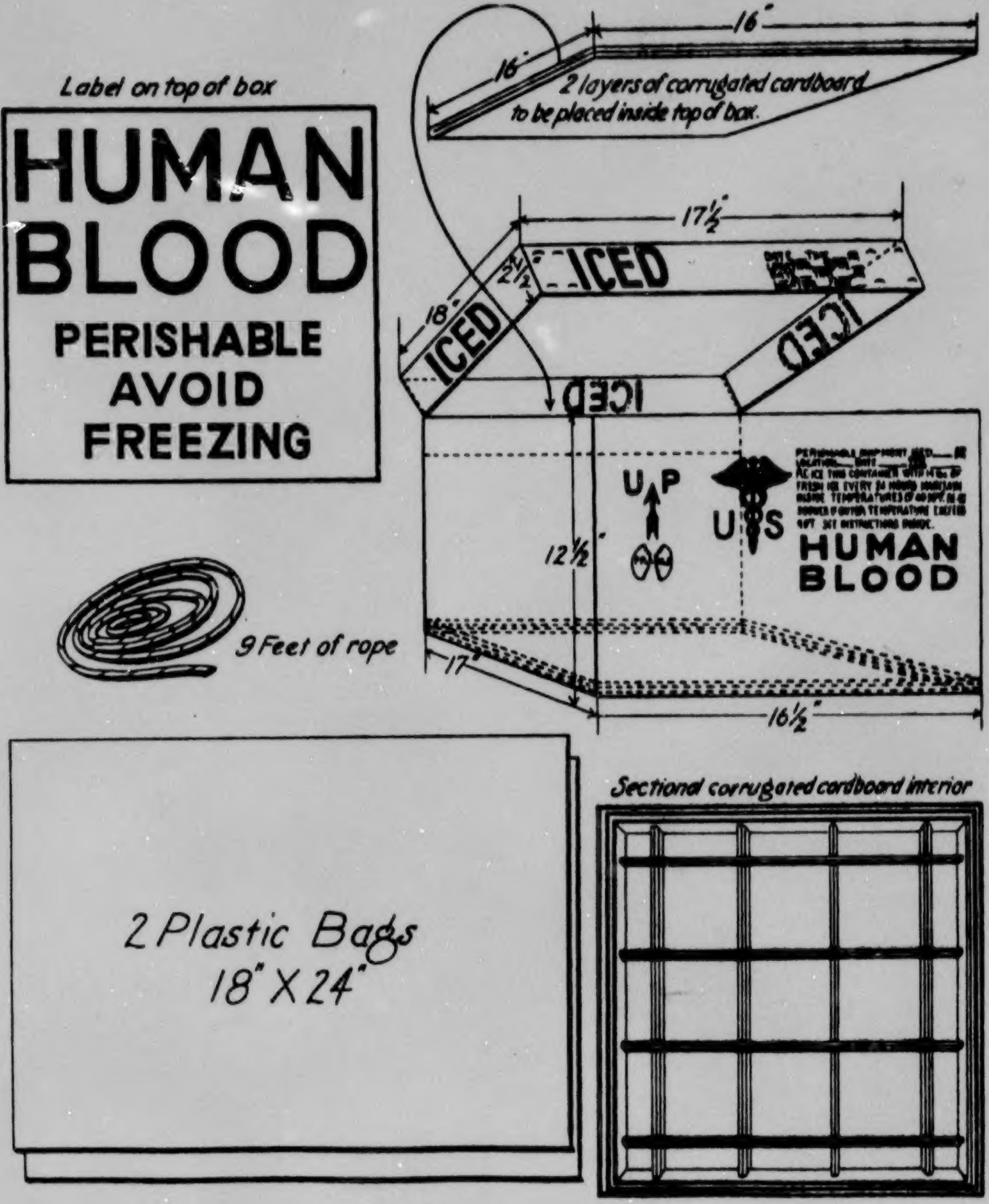
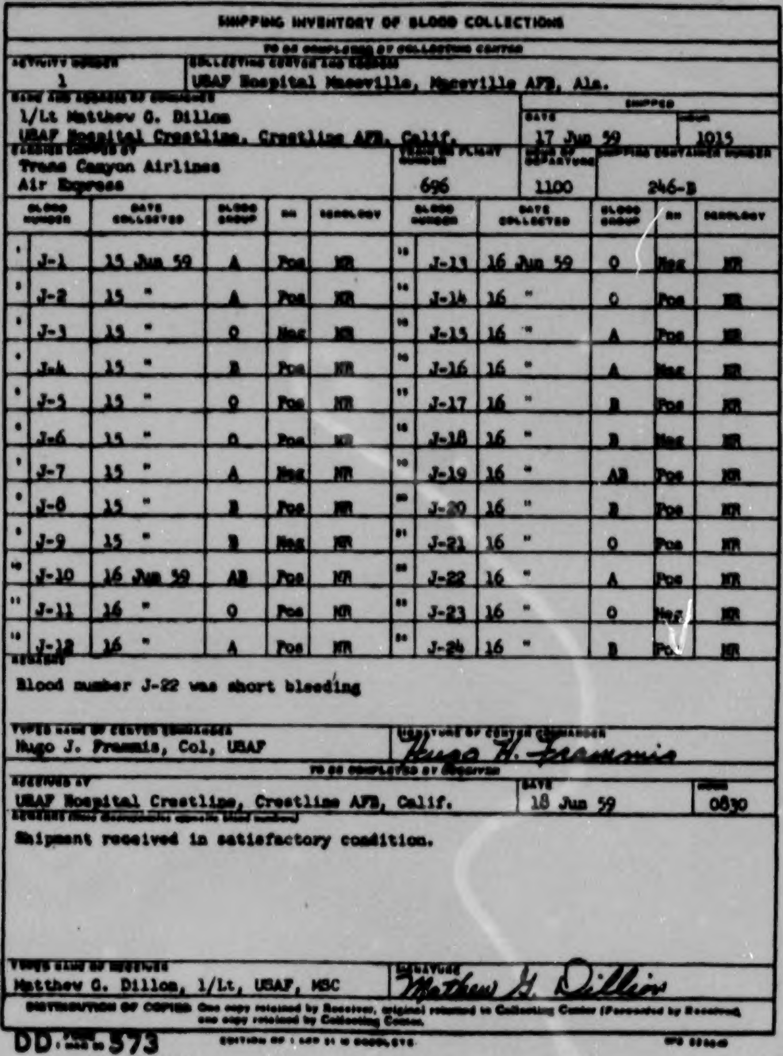

Label on top of box
HUMAN BLOOD
PERISHABLE
AVOID
FREEZING
16"
16"
2 layers of corrugated cardboard to be placed inside top of box.
17½"
2½"
18"
ICED
ICED
ICED
ICED
U P
U S
HUMAN BLOOD
12½"
9 Feet of rope
17"
16½"
2 Plastic Bags
18" X 24"
Sectional corrugated cardboard interior

Figure 10-25. Blood shipping carton

499



SHIPPING INVENTORY OF BLOOD COLLECTIONS

1

USAF Hospital Maceville, Maceville AFB, Ala.

1/Lt Matthew G. Dillon
USAF Hospital Crestline, Crestline AFB, Calif.

SHIPPED
17 Jun 59
1015

Trans Canyon Airlines
Air Express

696

1100

246-B

	BLOOD NUMBER	DATE COLLECTED	BLOOD GROUP	RH	SEROLOGY		BLOOD NUMBER	DATE COLLECTED	BLOOD GROUP	RH	SEROLOGY
1	J-1	15 Jun 59	A	Pos	NR	13	J-13	16 Jun 59	O	Neg	NR
2	J-2	15 "	A	Pos	NR	14	J-14	16 "	O	Pos	NR
3	J-3	15 "	O	Neg	NR	15	J-15	16 "	A	Pos	NR
4	J-4	15 "	B	Pos	NR	16	J-16	16 "	A	Neg	NR
5	J-5	15 "	O	Pos	NR	17	J-17	16 "	B	Pos	NR
6	J-6	15 "	O	Pos	NR	18	J-18	16 "	B	Neg	NR
7	J-7	15 "	A	Neg	NR	19	J-19	16 "	AB	Pos	NR
8	J-8	15 "	B	Pos	NR	20	J-20	16 "	B	Pos	NR
9	J-9	15 "	B	Neg	NR	21	J-21	16 "	O	Pos	NR
10	J-10	16 Jun 59	AB	Pos	NR	22	J-22	16 "	A	Pos	NR
11	J-11	16 "	O	Pos	NR	23	J-23	16 "	O	Neg	NR
12	J-12	16 "	A	Pos	NR	24	J-24	16 "	B	Pos	NR

Blood number J-22 was short bleeding

Hugo J. Frammis, Col, USAF

Hugo H. Frammis

USAF Hospital Crestline, Crestline AFB, Calif.

18 Jun 59

0830

Shipment received in satisfactory condition.

Matthew G. Dillon, 1/Lt, USAF, MSC

Matthew G. Dillon

DD 573

Figure 10-20. DD Form 573, "Shipping Inventory of Blood Collections."

carrier as to disposition. Blood should be returned to the center, if the delay is over 2 hours.

4. Blood containers should never be exposed to extreme temperature below freezing or over 85° F. Blood will not be placed in the belly compartment of planes or any place where the ambient temperature falls below 32° F.
5. If there is a delay en route, the carrier may be instructed to break the seal in order to reice. If this is done, a signed statement from the carrier agent breaking the seal, indicating the time and date reiced, must be placed in the container for the receiver's information. Wet ice only will be used for reicing.
6. The receiver will not accept a container of blood with a broken seal unless the explanation is signed by the carrier. In such case, the receiver should notify the officer in charge of the donor center immediately.

7. Blood will be transported as expeditiously as possible.
8. Every attempt must be made to have blood in the processing laboratory not later than the day after collection.
9. Processing laboratories will receive blood 24 hours a day, therefore, containers of blood should not be held overnight by the local carrier for delivery the next day.
10. Copies of the transportation instructions may be duplicated locally.

Special instructions on *SHIPMENT PROCEDURES UNDER DEPARTMENT OF DEFENSE BLOOD MOBILIZATION PLANS* are included in TM 8-255/NAVMED P-5004/AFM 160-16.

RECORDS IN THE BLOOD DONOR CENTER

Careful and accurate maintenance of records and labels is essential to the successful operation of a blood donor center. The minimum requirements in this regard are discussed below.

Donor Ledger

A suitable form for maintaining a record of blood donors is illustrated in figure 10-27.

Labeling of Collecting Bottles

All blood collected for shipment to processing laboratories shall be labeled and tied securely to the neck of the bottle or bag with a string. Do not use wire or rubber bands.

DD Form 572 (Blood Donor Record Card)

The pertinent facts obtained from the patient's history and physical examination will be entered on DD Form 572, which will show the initials of the technician who collected the blood and will be signed by the officer in charge of the blood donor center. All assigned numbers will be placed on DD Form 572 and the same identifying number will be placed on the bleeding container label, and pilot tubes.

DD Form 572 is the blood donor center's permanent record, which must be maintained on file. In event any question arises concerning the blood, the source of that blood may be traced to the donor. It is imperative that the release certificate on the reverse side be signed by the donor prior to collection of blood.

DD Form 573 (Shipping Inventory of Blood Collections)

This form, properly filled out, will accompany each shipping container. T[illegible] form contains spaces for the insertion of [illegible] blood donor's number, the date collected, and space for cause of rejection of blood after it reaches the processing laboratory. The blood donor center will fill out the upper portion of the form. The hour of ship-

BLOOD DONOR LEDGER

Date	Donor Number	Donor Name	Blood Group	Geno-type	Du Factor	Serol-ogy	Bili-rubin	Exp. Date

Figure 10-27. Example of form suitable for blood donor ledger.

ment is interpreted as the hour that the blood donor center seals the shipping container. The name of the carrier transporting the blood shipment and the train or flight number will be supplied by the transportation officer. DD Form 573 will be signed by the officer in charge of the blood donor center prior to shipment after the blood numbers indicated on form have been carefully checked and found to correspond exactly with the donor number stamped on each bottle or bag. The first three copies of DD Form 573 will be folded and placed in a manila envelope and forwarded inside the shipping container. Secure the manila envelope to the inside lid of the shipping container with tape. When the consignee receives the shipment, he will also verify the contents of the box as to their corresponding numbers on form. He will enter the place at which the blood was received, the date and the hour received. He will note discrepancies of individual bottles or bags opposite the blood numbers and make any pertinent remarks and sign form. One copy of DD Form 573 will be returned to the blood donor center that shipped blood. When the copy is received, the officer in charge of the blood donor center will note the discrepancies and remarks pertaining to this shipment and take the necessary corrective steps. This copy then will be matched with the copy retained by the blood donor center and filed as a permanent record.

EQUIPMENT FOR A BLOOD DONOR CENTER

Certain items of equipment are required for the operation of a blood donor center. These are listed below.

Reagents

Acetone.
Acid-Citrate-Dextrose (ACD) Solution, Formula A or B.
Alcohol, ethyl, 70 percent.
Copper sulfate solution (specific gravity 1.053 and 1.055 at 25° C.).
Tincture of iodine (3.5 percent and 7 percent).

Medications

Aromatic spirits of ammonia.
Coramine ampoules.
Caffeine sodium benzoate ampoules.
Epinephrine ampoules (1:1,000).
Procaine solution, 1 percent sterile.

Record Forms

Donor Ledger.
DD Form 572, (Blood Donor Record Card).

Materials and Equipment

Adhesive tape (one-half inch).
Applicator sticks, cotton-tipped, sterile.
Armboards.
Cylinders, glass.
Beds, or tables, flat (for collection of blood).
Blood bags.
Blood donor cart(s).
Blood lancets.
Blood shipping containers.
Bottle racks.
Capillary tubes.
Donor sets.
Dressings, sterile.
Forceps, sponge.
Gauze pads, 2 x 2 inch, sterile.
Needles, hypodermic, 26 gage, one-half inch.
Oxygen tank with face mask.
Pilot tubes, sterile (siliconized vacuum tubes are preferable).
Racks, test tube.
Refrigerator, to maintain constant temperature from 1° C. to 6° C. with a recording thermometer and alarm system. (If feasible, motor should not be attached to the box.)
Sphygmomanometers (snap cuff).
Sterilizer (dressing, laboratory, steam vacuum pressure).
Stethoscopes.
Stop watch (for timing hemoglobin determination).
Suture jars.
Syringes, Luer, 2 ml.
Tape, pressure sensitive (to secure pilot tubes to bottles).
Test tubes, Wassermann.
Thermometers, oral clinical.
Tincture of green soap.
Trays, syringe.
Vaccine bulbs (black rubber for capillary tubes).

Note. The amounts of the items listed above will depend upon the number of donors processed each day.

CHAPTER 11

BLOOD TRANSFUSION

Blood is composed of a complex fluid, plasma, which represents 55–60 percent of the total volume and suspended red cells, white cells, and platelets which comprise 40–45 percent of the total volume. Each of the cellular elements and each of the many components of plasma have specialized functions. The indication for transfusion of blood, therefore, should be based on the need for the special functions of the various components of blood.

In many instances the indication for blood transfusion is either for the erythrocytes or for the plasma alone, and one or the other of the components may be wasted when whole blood is given. If hemorrhage has occurred, restoration of both erythrocytes and blood volume may best be accomplished by administering whole blood. In the case of burns, crush injuries, and other forms of shock not accompanied by the loss of blood cells, the transfusion of plasma will restore the lowered blood volume as effectively and more safely than whole blood. In cases of anemia, not complicated by a diminished blood volume, erythrocytes alone will restore the desired red cell level more adequately than whole blood. The preparation and processing of each of these materials will be discussed.

TRANSFUSION OF WHOLE BLOOD

The most common of the blood transfusion procedures is the administration of whole blood. Whole blood is of special value where there is a massive and rapid loss of blood such as occurs in hemorrhage. It may also be employed to correct anemic conditions which are accompanied by a reduced blood volume or by shock. In addition, the physician may transfuse whole blood in bleeding disorders which are caused by defects in the blood clotting mechanism, and in exchange transfusion procedures (to be discussed later). The procurement of whole blood for transfusion purposes has been discussed in detail in chapter 10.

TRANSFUSION OF PACKED RED BLOOD CELLS

Concentrated packed red blood cells should be administered to anemic patients who are being prepared for elective surgery and who have a normal blood volume, since hemoglobin can be restored to the desired level in less volume as packed red cells. Additional units of cells may be given before or during surgery if they are required. In cases of shock, after the desired blood volume has been restored through the administration of plasma, anemia may be treated, if necessary, with the administration of concentrated cell suspensions. Packed red blood cells normally should not be used in the initial treatment of shock.

Cardiac patients may have anemia along with an increased blood volume. Effective blood volume is increased by plasma whereas the viscosity of the blood, but not the effective blood volume, is increased by red cells. When it is undesirable to further increase the blood volume, yet necessary to treat anemia, the administration of packed red blood cells is safest and most effective.

Erythrocyte suspensions contain a minimum of sodium chloride since the blood sodium chloride is contained in the plasma which is removed during the preparation of the cell suspensions. There is little sodium chloride in the medium (Alsever's solution) used for suspending the erythrocytes. In addition to the above factors overload reactions are significantly less in number when erythrocyte suspensions are transfused in place of whole blood.

Medical patients may require transfusion of packed red blood cells in (1) chronic hemorrhage (normal blood volume), (2) iron deficiency anemia, (3) nutritional anemia, and (4) anemia of pregnancy.

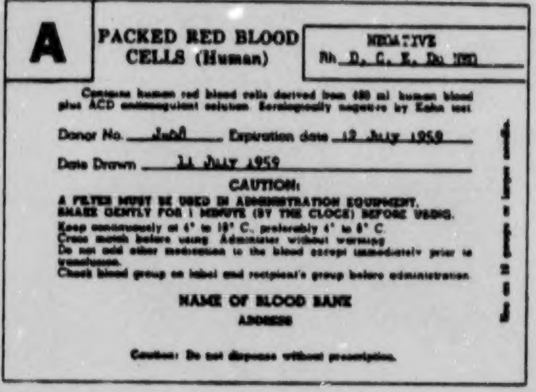
When whole blood is administered for the treatment of the above conditions, the plasma will be wasted. Even in the case of nutritional anemia, the quantity of protein which can be transfused in whole blood is so small that it has little therapeutic effect. The protein requirements of the patient are best supplied through diet.

Preparation of Packed Red Blood Cells

Plasma should not be separated from whole blood until the physician orders a unit of packed red cells for transfusion. In choosing an appropriate unit for packed cell suspension the freshest possible unit of compatible blood should be used to avoid hemolysis.

The detailed procedure for preparing packed red blood cells is outlined below.

1. With a single plastic unit (where the hermatic seal is broken) packed cells must be used within 24 hours.
    - *a.* Choose a compatible unit in which sedimentation is complete, or centrifuge blood pack unit, under 6 days old, 1,500 to 2,000 r.p.m. for 20 minutes (standard centrifuge).
    - *b.* Place two loose, overhand knots in transfer pack tubing and place clamp on tightly.
    - *c.* Open coupler, pull apart tab exposing sterile field.
    - *d.* Insert coupler, puncturing the plastic diaphragm and into the membrane seal.
    - *e.* Release clamp on transfer pack. With either mechanical pressure or a plasma extractor, force plasma into transfer pack until plasma cell interface reaches proximal part of tubing and clamp off.
    - *f.* Close clamp tightly.
    - *g.* Pull the two knots tightly (white-tight) and sever between the knots to separate units.
2. With a multiple blood pack unit having either double, triple, or quadruple blood pack unit, whole blood drawn in ACD with the plasma removed in a closed (nonvenous) system with sterility maintained is good for the 21-day expiration dating period.
    - *a.* Choose a compatible unit in which sedimentation is complete, or centrifuge blood pack unit, under 6 days' old, 1,500 to 2,000 r.p.m. for 20 minutes (standard centrifuge).
    - *b.* Place two loose, overhand knots in the transfer pack tubing and place clamp on.
    - *c.* Force the shot-like head in the transfer pack into the pack. Open clamp.
    - *d.* By pressure either with a plasma extractor or with mechanical pressure, force plasma into the transfer pack, until plasma cell interface reaches proximal part of tubing, and clamp off.
    - *e.* Close clamp tightly.
    - *f.* Pull the two knots tight (white-tight) and sever between the knots to separate units.



A

PACKED RED BLOOD CELLS (Human)

NEGATIVE
Rh D, C, E, Du [illegible]

Contains human red blood cells derived from 480 ml human blood plus ACD anticoagulant solution. Serologically negative by Kahn test

Donor No. [illegible] Expiration date 12 July 1959

Date Drawn 11 July 1959

CAUTION:

A FILTER MUST BE USED IN ADMINISTRATION EQUIPMENT.
SHAKE GENTLY FOR 1 MINUTE (BY THE CLOCK) BEFORE USING.
Keep continuously at 4° to 10° C., preferably 4° to 6° C.
Cross match before using. Administer without warming.
Do not add other medication to the blood except immediately prior to transfusion.
Check blood group on label and recipient's group before administration.

NAME OF BLOOD BANK
ADDRESS

Caution: Do not dispense without prescription.

Figure 11-1. Label for packed red blood cells.

PLASMA TRANSFUSIONS

The liquid portion of blood plasma possesses a variety of different substances. These include (1) fibrinogen, thrombin, and accessory factors involved in the clotting of blood; (2) antibodies which are contained in the globulin fraction of plasma; (3) various crystalloids and electrolytes; and (4) most important from the standpoint of transfusion, the blood colloids (largely albumin) which play a part in the maintenance of a normal blood volume.

Variations in blood volume are related to the condition known as shock. There are two important clinical types of shock. The first is neurogenic shock which appears rapidly and is set in motion by the nervous system. It is characterized by a sudden decrease in the blood pressure, a slow pulse in the early stages, the dilation of the blood vessels. This last factor increases the volume capacity of the vascular system which creates a relative loss of effective blood volume. In the therapy of neurogenic shock, agents such as adrenalin or other stimulants (by effective vasoconstriction) will restore the circulation by decreasing the volume capacity of the vascular system. Usually, it is unnecessary to give fluids to combat neurogenic shock.

The second form of shock is called hematogenic shock and is generally associated with bodily injury in which tissues are damaged or blood is lost. This condition is characterized by a more gradual appearance of symptoms and a more slowly falling blood pressure. The chief characteristic of hematogenic shock is a decreased blood volume and a diminished volume capacity in the vascular system because of the compensatory vasoconstriction. The pulse in this form of shock is usually rapid.

The treatment of hematogenic shock requires the restoration of the blood volume by the rapid infusion of fluids containing colloids which may be held in the vascular system. In hematogenic shock the circulation fails because of a reduction in effective blood volume. The capillaries lose their ability to retain colloidal material and this vital element of blood is lost through the capillaries into the tissues. Blood plasma is the effective component of whole blood instrumental in combating and correcting this form of shock. The red cells of whole blood do not possess these shock combating properties. If there has been a great and rapid loss of erythrocytes as well as plasma, whole blood is necessary to restore the normal condition. Even in shock due to blood loss, however, the blood volume and thus the circulation may be restored with plasma alone, and in emergency a life may be saved.

In addition to correcting decreased blood volume, plasma has other uses. It is important as a source of blood clotting components and immune antibodies. Generally speaking, these are labile substances and will lose their potency if stored at room temperature for a long period of time. Special pools or single units of plasma should be prepared from freshly collected blood and frozen as quickly as possible if it is desired to administer plasma for the correction of clotting deficiencies or to utilize the immune antibodies. In brief, the indications for the use of plasma include (1) shock due to loss of blood, (2) dehydration, (3) shock due to burns and crush injuries, (4) clotting deficiencies, and (5) need for immune antibodies.

Preparation of Plasma

Plasma or serum for transfusion must be prepared by a closed or vented method under sterile conditions. The methods employed must yield a bacteriologically sterile and pyrogen free product. The detailed procedure for preparing plasma is outlined below.

1. Whole blood collected into 4 percent sodium citrate is preferred for the preparation of plasma because (a) a greatly increased yield of plasma is obtained and (b) the fibrinogen in the plasma has greater stability than in plasma separated from whole blood drawn in ACD.
2. The blood is stored at refrigerator temperature for 24 hours after its withdrawal from the donor.
3. In order to obtain the maximum yield of plasma, the blood is centrifuged for 1 hour at 2,000 r.p.m. With this amount of centrifugation the erythrocytes are tightly packed and a good separation of plasma results.
4. The aspirating set is sterile and consists of—

a. A long blunt-tipped aspirating needle.

b. A disposable sterile tube equipped with a control valve or hemostat.

c. A large needle to be placed in a 2 liter, evacuated, pooling bottle containing 200 ml. of sterile pyrogen free, 50 percent glucose solution.

5. The tops of the pooling bottle and the blood collection bottle must be carefully cleaned by coating with 7 percent tincture of iodine before the needles fastened to the aspirating set are inserted through the stoppers into the bottles. The valve or clamp on the tube must be closed before the pooling bottle is entered.

6. The long aspirating needle is inserted through the stopper of the blood collection bottle. After the needle is in position, the hemostat or clamp is released and the plasma is permitted to flow into the pooling bottle.

7. Considerable practice is required to aspirate maximum quantities of plasma from the blood collection bottle without disturbing the sedimented erythrocytes. If cells are aspirated over into the plasma, the material should be recentrifuged and the erythrocytes removed.

8. The hole which is made when the aspirating needle is inserted through the thin plug of the blood bottle stopper should be closed aseptically. Tight fitting sterile hoods are quite useful for this purpose.

9. Pools are made to contain the plasma from 3 units of group O, 2 of group A, and 1 of group B blood. This pooling is planned so that the soluble A and B antigens present in group A and B plasma will lower the titer of anti-A and anti-B agglutinins present in the pooled plasma.

10. Each pool of plasma contains 6 units (approximately 300 ml. each) and 200 ml. of 50 percent glucose. This provides a 10 percent dilution of the plasma and increases the glucose content by 5 percent. The additional glucose contributes greatly to the stability of plasma stored at room temperature.

11. Twenty ml. of sterile 1 percent merthiolate is added to complete the pool of plasma. This small quantity of chemical preservative will be effective in preventing the growth of chance airborne contaminating organisms. Merthiolate in this concentration has been demonstrated to be harmless to patients.

12. The completed pool should be allowed to stand for 14 days at room temperature and observed for the presence of turbidity which may indicate the growth of bacteria.

13. After the 14-day standing period, a specimen of the plasma is withdrawn for titration against known A and B cells. The titer of the pooled plasma against each of these cell groups must be below 1:32. If the antibody titer is found to be above 1:32, the pool must be divided and either low titer A and/or B plasma or sterile group specific substances added to reduce the isohem-agglutinin content.

14. At this same time a 10 ml. sample of the pooled plasma is withdrawn and inoculated into NIH Sterility Media (fluid thioglycollate).

15. The sterile plasma pool (demonstrated to be sterile on the basis of culture) is divided into 300 ml. or 600 ml. bottles. If the plasma has been processed from blood drawn in ACD solution, a 400 ml. unit is prepared as equivalent to the plasma from 500 ml. of whole blood. A 300 ml. unit of plasma from sodium citrated blood is equivalent to plasma from 500 ml. of whole blood.

16. The individual bottles from each plasma pool are held at room temperature for 14 days at which time cultures are prepared from a pilot bottle. The pilot bottle contains the last 50 mls. of plasma from the pool and is set up at the time the pool is divided into individual bottles.

17. These cultures are held for 14 days and must be sterile at the end of this period.

18. Each bottle is clearly labeled. Liquid plasma has an expiration date of 2 years from the date of collecting the oldest blood in the pool. It is recommended that fluid plasma be stored at a temperature above 25° C. for 6 months before issue to

reduce or eliminate the risk of transmitting viral hepatitis.

19. Plasma that has been demonstrated to be sterile on the basis of culture is segregated until one bottle from the pool is given to a patient. If there is no transfusion reaction to this first bottle of the pool, the entire pool is cleared for use. This is called a human pyrogen test. If a patient reaction does occur following the use of the pooled plasma, the pool should be discarded.
20. The remainder of the contents of the pilot bottle is held for study until all bottles in the pool have been used.

Use of Frozen Plasma

The labile components of human plasma will usually disappear during the long storage period required to free fluid plasma from the risk of transmitting viral hepatitis. For those individuals who require antihemophiliac globulin, fibrinogen and other labile plasma components, it is best to prepare single units of nonpooled plasma. These units should be labeled according to blood group and donor number.

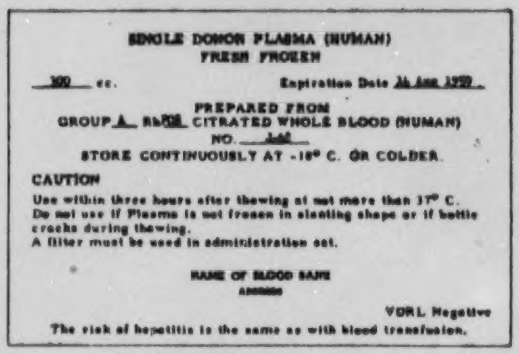
The plasma should be aspirated shortly after bleeding of the donor and centrifugation of the container. It should be collected into a sterile, pyrogen free empty vacuum bottle. The plasma should be stored at −20° C. or lower. It should be thawed at 37° C. just before it is administered. This use of single donor, fresh or frozen plasma tends to reduce the risk of transmission of viral hepatitis. It is advisable to maintain at room temperature for 10 minutes prior to placing in 37° C. waterbath.

Labeling of Plasma Containers

Labels for plasma containers must include the name of the product, i.e., "Normal Human Plasma." In addition, the information presented below must appear on the label.

1. The volume of plasma represented in the original pool.
2. The volume of plasma actually contained in the bottle.
3. The amount of glucose that has been added.
4. A statement concerning the presence or absence of preservatives. If preservatives have been added, the final concentration must be indicated.
5. A statement in a prominent position that the plasmas must be stored at 15°–30° C. (room temperature).
6. A statement of caution concerning the storage and administration of the plasma. The cautions are indicated below.
   - *a.* A filter must be used in the administration of this plasma.
   - *b.* Do not use this plasma if it is definitely turbid or discolored, unless due to the formation of fibrin. Fibrin formation or a granular deposit may normally develop with aging.
   - *c.* Plasma should not be warmed before administration.
   - *d.* Do not store in the refrigerator.
7. The identifying lot number and expiration date which is established from the date of the preparation of the final plasma pool. The period is 2 years for liquid human plasma.
8. The name and address of the blood bank.

Typical labels for plasma containers are illustrated in figures 11-2 and 11-3.



SINGLE DONOR PLASMA (HUMAN)
FRESH FROZEN

300 cc. Expiration Date [illegible]

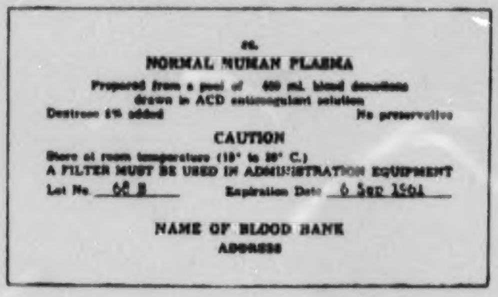
PREPARED FROM
GROUP A Rh [illegible] CITRATED WHOLE BLOOD (HUMAN)
NO. [illegible]
STORE CONTINUOUSLY AT -18° C. OR COLDER.

CAUTION
Use within three hours after thawing at not more than 37° C.
Do not use if Plasma is not frozen in slanting shape or if bottle cracks during thawing.
A filter must be used in administration set.

NAME OF BLOOD BANK
ADDRESS

VDRL Negative
The risk of hepatitis is the same as with blood transfusion.

Figure 11-2. Label for fresh frozen plasma.



cc.
NORMAL HUMAN PLASMA
Prepared from a pool of 400 ml. blood donations drawn in ACD anticoagulant solution
Dextrose 5% added No preservative

CAUTION
Store at room temperature (15° to 30° C.)
A FILTER MUST BE USED IN ADMINISTRATION EQUIPMENT
Lot No. 60 B Expiration Date 6 Sep 1961

NAME OF BLOOD BANK
ADDRESS

Figure 11-3. Label for pooled normal human plasma.

PLATELET TRANSFUSION

Platelets are often decreased in number in patients suffering with idiopathic thrombocytopenic purpura, or individuals with aplastic anemia, luekemia, etc. Some patients possess antiplatelet antibodies which are routinely difficult to detect. Fresh blood drawn into siliconized glass bottles or plastic bags and immediately administered to patients who require whole blood, probably provide the most adequate platelet therapy. When such platelets are given to recipients who have antiplatelet antibodies, however, few circulating platelets survive. With the advent of plastic containers for whole blood, it is possible to concentrate platelets by centrifugation into small volumes of plasma. The procedure is as follows:

1. A unit of the appropriate group and type of fresh whole blood is drawn from a suitable donor.
2. The unit of whole blood is centrifuged at approximately 1,000 r.p.m. for 15 minutes, to adequately pack down the red cells and create a buffy coat.
3. The supernatant plasma and buffy coat containing both leukocytes and platelets are transferred to a secondary plastic pack.
4. The secondary plastic pack is then centrifuged at approximately 2,000 r.p.m. for 20 minutes. This centrifugation results in an aggregation of platelets in the bottom of the second transfer pack.
5. A supernatant plasma is withdrawn from the secondary plastic pack. About 30 to 50 cc. of plasma is left in the secondary transfer pack and the platelets contained there are resuspended.
6. This concentrated platelet transfusion pack is then administered to the patient through siliconized needles and tubing.

Using these methods, it is possible to transfer great quantities of concentrated platelets with the addition of minimal quantities of fluid into a critically ill patient.

PLASMA AND CELL SEPARATION USING PLASTIC BLOOD CONTAINERS

The procedures to be described below employ a plastic blood container (fig. 11-4) and a plastic transfer pack (fig. 11-5). They are intended for the aseptic subdivision of whole blood into smaller units, for the separation of plasma from cellular elements, for the preparation of packed cells and the preparation of platelet-rich plasma or platelet concentrates.

Sedimentation

Sedimentation of red cells in ACD blood in a plastic blood pack begins shortly after collection. After 24 hours in the refrigerator, the red cells will have become well concentrated, and little further settling will occur thereafter (fig. 11-6). The supernatant plasmas will approximate 60 percent of the total plasma plus anticoagulant solution. The residual cell suspension will then contain about 60 percent of cells by volume (hematocrit = 60 – 70). A high percentage of the white cells and platelets will have settled out in a layer above the red cells, the so-called buffy coat. Sedimentation is therefore not recommended as a method for harvesting platelets.

Centrifugation

Centrifuge cups for 650 ml. bottles will accommodate all plastic blood packs for 500 ml. of blood (fig. 11-7) and the 600 ml. and 300 ml. transfer packs. Cups for the 250 ml. bottles will accommodate a 150 ml. transfer pack. The plastic blood pack or transfer pack must be fairly well filled with fluid to prevent folding over of the top of the container and the trapping of cellular elements above the fold. The attachment of a clamp to an outlet tube can aggravate this condition. To prevent excess distortion of the bottom of the plastic blood pack, the centrifuge cup should contain water to a depth of about 1 inch before the unit is placed in the cup. The water will then rise to a level which will safely support the bottom part of the blood pack. Additional water for balancing is added after the units are placed in the cups (fig. 11-8). When supported with a water cushion in this manner, the blood packs and transfer packs can withstand centrifugation at maximum centrifuge speed. The centrifugation speeds recommended for component separation are as follows:

1. Platelet-rich plasma—600 r.p.m. for 15 minutes.
2. Platelet concentrate—1,500 r.p.m. for 20 minutes.
3. Plasma free of cellular elements—2,500 r.p.m. for 20 minutes.

Assembly of Equipment

To assemble a transfer pack to the blood pack for the aseptic separation of whole blood or blood components, the procedure outlined below should be followed:

Note. This procedure, as presented, employs a unit of equipment called a plasma extractor. A plasma extractor is not required, however, for satisfactory separation of cells and plasma. Manual pressure has proven quite adequate where a plasma extractor is not available.

1. When dividing whole blood, mix the plasma and cells thoroughly by inverting the blood pack at least a dozen times. When separating components, do not disturb the plasma red cell interface.
2. Throw two loose overhand knots over the inlet tubing of the transfer pack. Loosen the coupler cover but do not remove (fig. 11-9). Expel the air from the transfer pack and close the clamp tightly on the tubing.

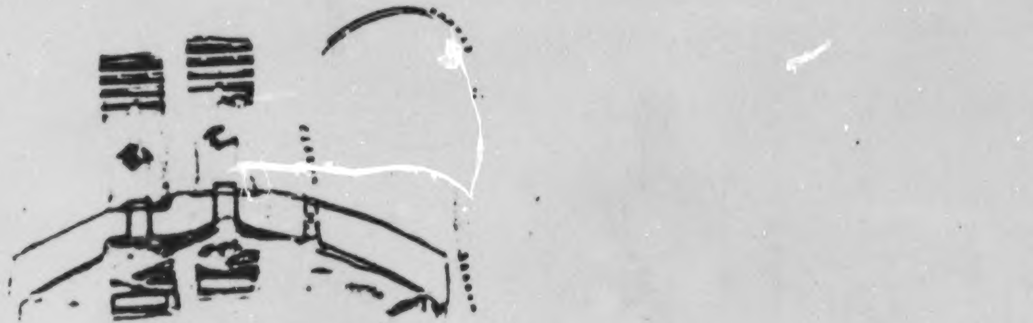


Figure 11-4. Plastic blood container.

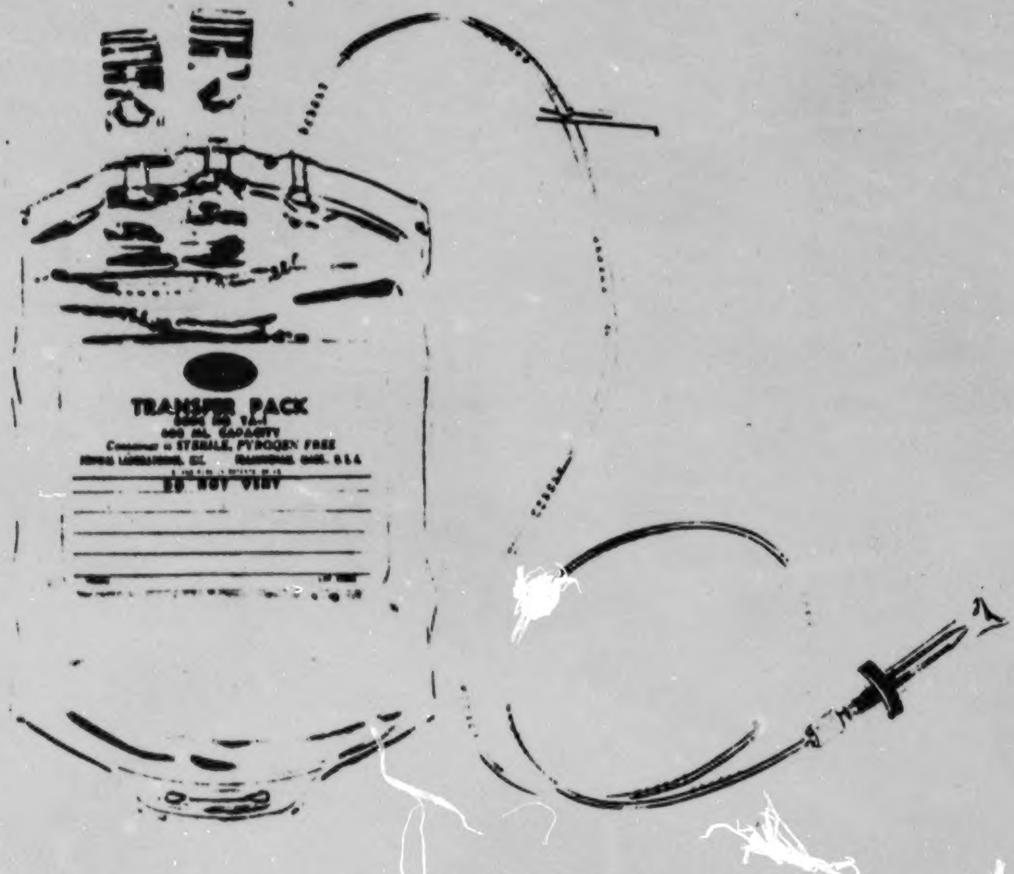


Figure 11-5. Plastic transfer pack.

3. Grasp the tabs of the center outlet port on the selected blood pack, pull them apart and fold them back to expose the sterile outlet tube (fig. 11-10).

4. Hold the blood pack with the thumb and forefinger over the folded tabs (fig. 11-11). Remove the cover from the coupler of the transfer pack. Insert the coupler into the outlet tube and twist it all the way up the flange, puncturing the plastic diaphragm of the port (fig. 11-12).

5. Suspend the blood pack in the plasma extractor, engaging the holes in the upper seal with the studs on the extractor (fig. 11-13). The transfer pack is than laid or suspended alongside the plasma extractor.

6. Release the clamp on the tubing.

7. Disengage the handle of the plasma extractor and gradually apply pressure to the blood pack by continuous slow advance of the movable panel. By observation of the interface, the expression of a component into the transfer pack is continued until the first part of the tubing shows complete expression accomplished.

8. Close the clamp tightly on the tubing.

9. Re-engage the handle of the plasma extractor in the hook to lock it in an open position.

10. Pull the two knots in the transfer pack tubing white-tight and sever between the knots to separate the units. The above procedure applies for the division

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Figure 11-6. After 24 hours in the refrigerator, the red cells become well concentrated, and little further settling occurs thereafter.

Figure 11-7. Centrifuge cups for 650 ml. bottles will accommodate all plastic bloodpacks for 500 ml. of blood as well as the 600 ml. and 300 ml. transfer packs.

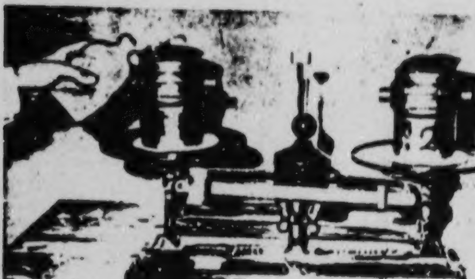

Figure 11-8. Additional water for balancing is added after the units are placed in the cups.

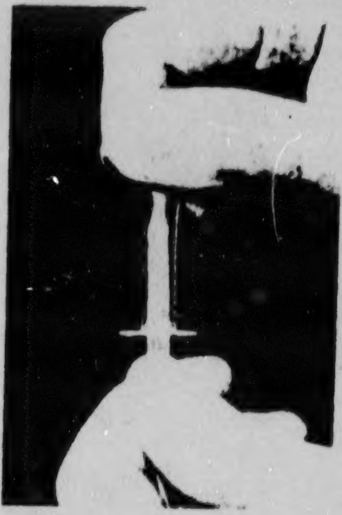

Figure 11-9. Loosen the coupler cover but do not remove.

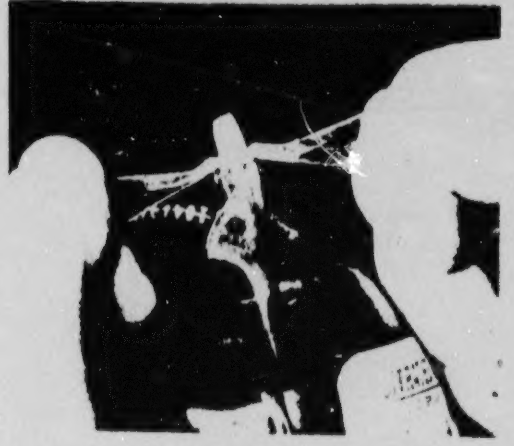

Figure 11-10. Grasp the tabs of the center outlet part on the selected blood pack, pull them apart and fold them back to expose the sterile outlet tube.

of blood or for the separation of platelet-rich plasma or red cells. When a secondary centrifugation is necessary, as in preparing a platelet concentrate, the two knots in the transfer pack tubing are not pulled tight, but both units are returned to the centrifuge cup in an upright position.

11. Centrifuge at the recommended speeds.
12. The transfer pack is placed in the plasma extractor and the supernatant plasma expressed into the primary container and

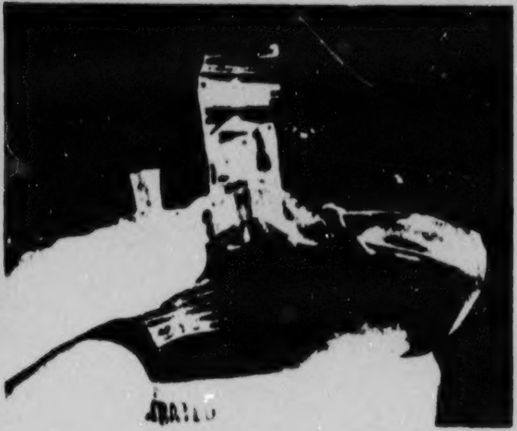

Figure 11-11. Hold the blood pack with the thumb and forefinger over the folded tabs.

mixed with the red cells. Then the two knots are pulled white-tight and the units separated by severing between the knots.

13. If the supernatant plasma is not to be returned to the primary container, the transfer pack tubing is divided between the two white-tight knots.
14. If a platelet concentrate is to be prepared, the transfer pack is recentrifuged at the recommended speed.
15. The supernatant plasma is then expressed into another transfer pack as described in steps 1–10, inclusive, leaving the platelets in a small volume (15–20 ml.) of plasma in the primary transfer pack. Red cells separated as described above may then be returned to refrigerated storage but must be used within 24 hours after collection. Platelet-free plasma can be infused fresh, or stored in the liquid or frozen state, or pooled.

Figure 11-12. Remove the cover from the coupler of the transfer pack. Insert the coupler into the outlet tube and twist it all the way up to the flange, puncturing the plastic diaphragm of the port.

UNIVERSAL DONOR TRANSFUSIONS

Inasmuch as group O blood possesses no agglutinogens (International) in the erythrocytes (this has been subject to question within recent years), it has been generally known that group O blood usually can be transfused into patients who are groups A, B, or AB, without apparent danger. This practice gained ascendency under wartime conditions when group O blood was administered to thousands of casualties without grouping the recipient or crossmatching.

There is a certain degree of individuality in genetic structure of the blood antigens of each member of the human race. These involve almost infinitely variable combinations of factors of the ABO, Rh-Hr, Kell, MNS, Lutheran, Duffy, Kidd, Lewis, P, Levay, and other systems, in addition to systems not yet recognized. Our knowledge of the long range immunologic effects of transfusion is incomplete, and is being expanded continually by constant research, observation, and case reports. At the present time it appears the wisest course to follow, sofar as possible and practicable, is to transfuse a patient with blood from a donor whose blood factors are as nearly similar as is obtainable; i.e., group and type specific.

The practice of routinely and indiscriminately transfusing all recipients with group O blood, regardless of groups, is advocated by almost no one. There are, nevertheless, instances where use of group O, Rh negative, blood as a universal donor is not only advisable but also necessary. The chief indication for use of group O, Rh negative, blood without regard to the recipient's grouping and typing is in instances of massive hemorrhage with attendant shock. In these conditions life is in immediate danger and delay

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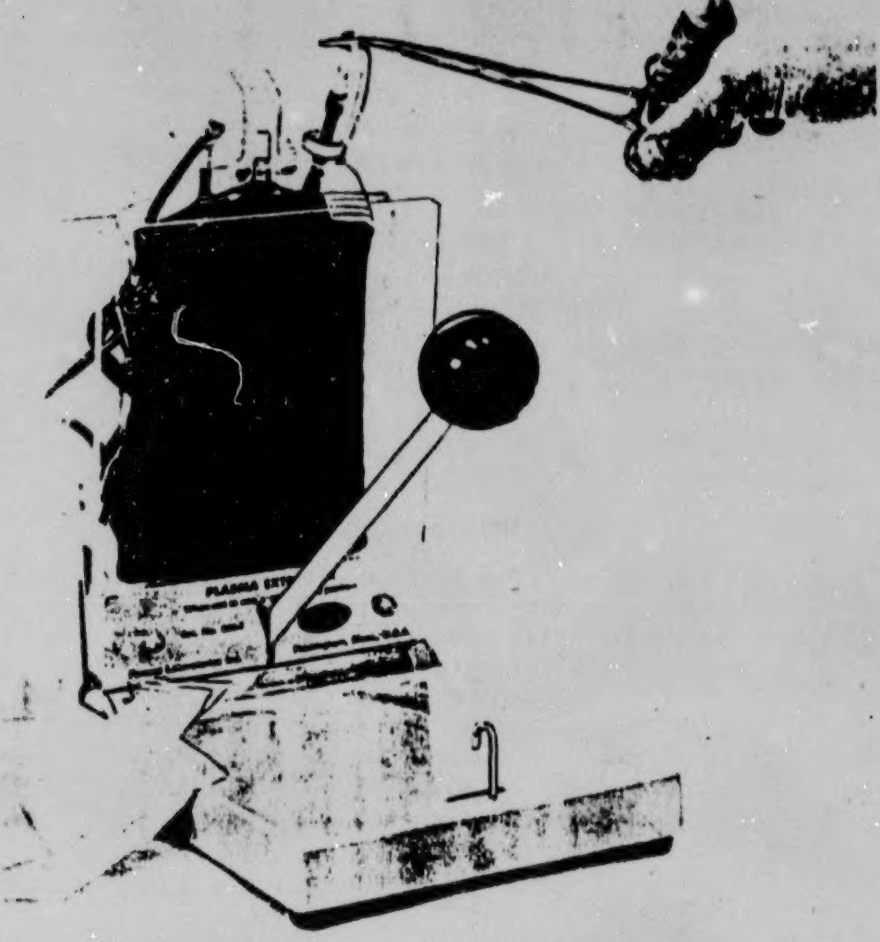

Figure 11-13. Suspend the blood pack in the plasma extractor, engaging the holes in the upper seal with the studs on the extractor.

occasioned for purposes of typing and crossmatching would in all probability result in death.

Much has been written and discussed regarding the danger of a hemolytic transfusion reaction resulting from a high titer of anti-A or anti-B agglutinins in group O plasma combined with a high degree of cell sensitivity in patients of groups A, B, or AB. Admittedly there is always an element of risk involved in this situation, as indeed there is a calculated risk in performing any transfusion at all. Failure on the part of blood bank personnel to expedite administration of available group O blood in an emergency (when in the physician's judgment the patient's survival is immediately at stake) for fear of the aforementioned reaction, is inexcusable. The chance of untoward reactions resulting from a group O transfusion weighed against the immediate danger to life from hemorrhage and shock is a matter of medical judgment and is not the responsibility

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of blood bank personnel. It is, however, the responsibility of blood bank personnel to maintain as adequate a supply of selected screened low-titered group O, Rh negative, blood as is possible at all times in anticipation of such unusual emergencies. Criteria for selection of these group O bloods were discussed in chapter 10. Prior to releasing group O, Rh negative, blood for use in such emergencies, rapid confirmation of grouping and typing is of course an obvious essential.

Recent studies indicate the danger of giving large transfusions of universal donor blood to patients of group A, B, or AB, and following this therapy by giving group specific blood within 2 weeks.

When a patient has received a transfusion of group O blood in excess of 4 pints and requires subsequent transfusions within a period of 2 weeks he should receive group O blood regardless of his heredity group.

Emergency cases receiving multiple units of group O blood prior to specific grouping represent a special problem. Complete and accurate records are necessary on such individuals. Certainly pretransfusion samples of the recipient's blood should not be used in any crossmatch tests after the infusion of 4 pints of blood. It is safer to obtain a post-transfusion sample from the recipient for crossmatching. The recipient's blood may contain iso-antibodies—from these recent transfusions or from a previous sensitization—capable of destroying some donor's cells.

BLOOD GROUP SPECIFIC SUBSTANCES A AND B (WITEBSKY)

Instances of mishaps resulting from the use of universal donor group O blood have been reported occasionally, usually from the harmful effect of anti-A and anti-B agglutinins in group O plasma upon the erythrocytes of recipients of groups A, B, and AB. In order to avoid these difficulties, attempts have been made to neutralize these agglutinins by the addition of purified group A and B specific substances derived from hog and horse stomachs. This product is available in a 10 ml. vial for injection into the blood unit, immediately prior to transfusion, according to the manufacturer's specifications.

It has been shown that satisfactory reduction of anti-A and anti-B titer to low levels can be changed by this means in most instances. Recently, however, it has been demonstrated that there are immune forms of anti-A and anti-B iso-agglutinins which are largely unaffected by the injection of the Witebsky Group Specific Substances. It is advisable, therefore, to determine the titer of immune anti-A and anti-B antibodies. Any units of group O, Rh negative, blood showing an unsatisfactory titer should be rejected for universal donor purposes.

It should also be conceded that there is not complete agreement on the advisability of injecting Witebsky substance. From the immunologic viewpoint there are possible theoretical objections, especially from the standpoint of a female recipient of childbearing age. In this instance it is conceivable that transfusions with group O blood with A and B specific substances added may result in a high immune anti-A or anti-B antibody titer. This possibly could produce potential risk of damage to a heterologous fetus in a subsequent pregnancy. Should there be potential erythroblastosis due to ABO sensitization in a subsequent pregnancy, this procedure possibly could increase the risk of this potentiality becoming actual.

EXCHANGE TRANSFUSIONS

Erythroblastosis fetalis (hemolytic disease of the newborn) is due to the presence of antibodies circulating in the plasma of the infant, that were produced in the mother and crossed the placenta. These antibodies are produced because of antigens present in the fetus' erythrocytes and absent in the mother's. Some fetal erythrocytes escape the fetus into the maternal circulation and produce sensitization or antibody formation.

The antibodies may be anti-A, anti-B, anti-Rh, anti-Kell, or other factors. The presence of these antibodies causes hemolysis of the infant's erythrocytes, hence producing anemia and hyperbilirubinemia. If the antibody titer is low, the disease may be mild and not treatment required; however, if the titer is high and anemia and/or hyperbilirubinemia severe, then exchange transfusion is necessary.

The purpose of the exchange transfusion is to—

1. **Replace destroyed erythrocytes.**
2. **Remove antibodies to the infant's red blood cells.**
3. **Remove some small amount of bilirubin, but mainly to prevent a further rise in bilirubinemia. (In severely affected infants the large amounts of bilirubin present in tissues, aside from the blood compartment producing jaundice, cannot be markedly influenced by exchange transfusions.)**

Exchange transfusion performed by alternately withdrawing blood from the infant and replacing it with an equal amount of fresh donor blood. This is done in 10 to 20 cc. increments until 300 to 600 cc. of blood is replaced, depending on the size of the infant. The exchange is usually carried out through the umbilical vein: however, other vessels may be used.

In selecting the type of blood for exchange transfusion, the most important consideration is that the fetus produces little or no natural antibodies, hence, most of the circulating antibodies present at birth are from the mother and any blood administered must be compatible with the mother's serum.

The cardinal rules governing the choice of blood for exchange transfusion are—

1. Avoid the antigen responsible for the antibodies present in the mother's serum.
2. Administer blood with phenotype specific to mother as to Rh, Kell, etc. and
3. Use group specific cells compatible with mother and child or group O with low titer serum.
4. Use fresh blood, preferably under 24 hours old and never more than 72.

REQUISITIONING OF BLOOD

Whenever possible, transfusions should be planned in advance. Not only are errors more likely to occur when technicians are working under the pressure of little time, but the time required for adequate crossmatching can hardly be reduced below 1½ hours from the time that the physician orders the blood to the time it is ready for transfusion. Lastly, requisitioning of "STAT" blood at night should be resorted to only when an actual emergency exists.

Standard Form 518 (Clinical Record-Blood Transfusion)

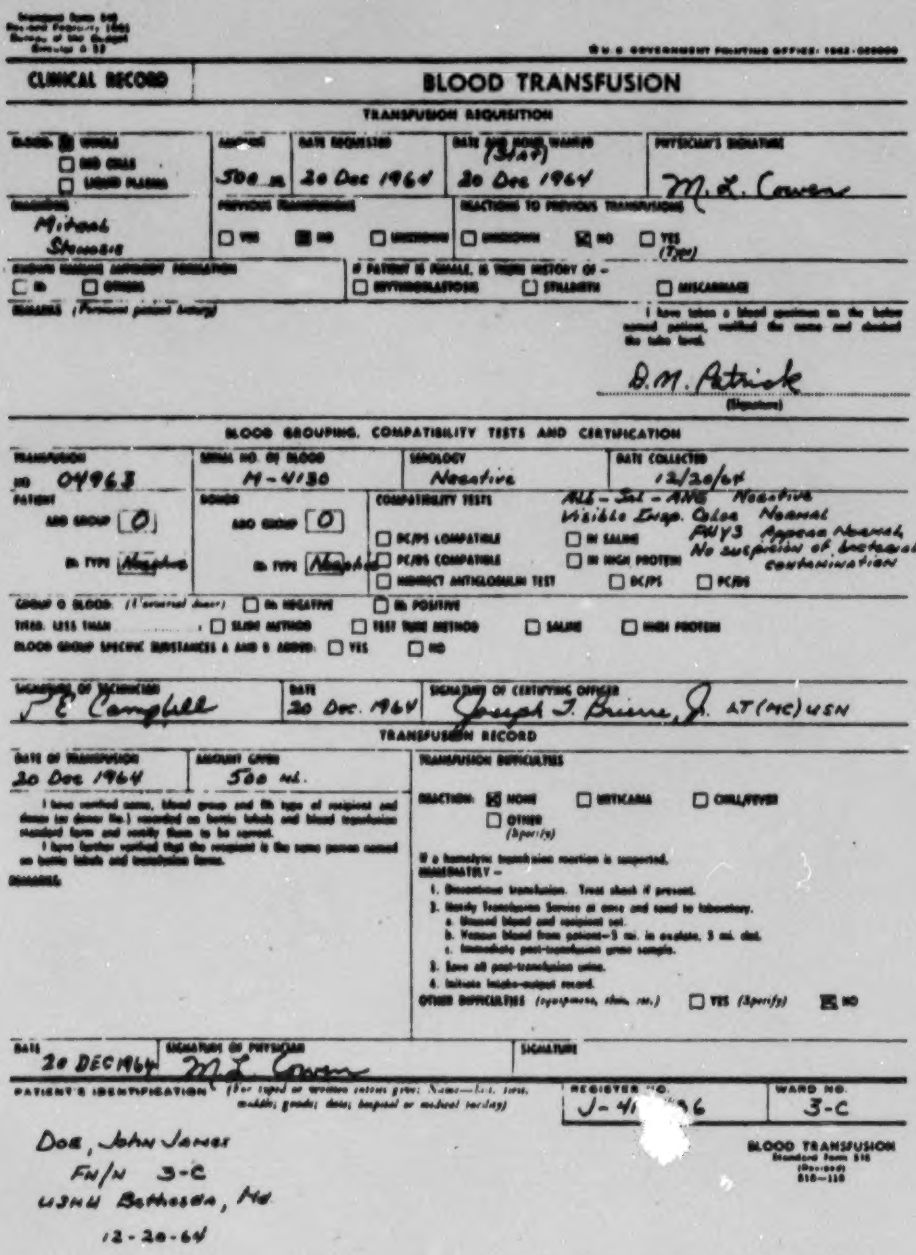
Requests for blood should be submitted on SF 518 (fig. 11-14). Requests in triplicate for each 500 ml. of blood or a portion should be completed by ward or operating room personnel and delivered to the blood bank. The request should state the amount, where it is to be used (in which ward or operating room), date and hour needed, and signature of the requesting physician. All information pertinent to the patient should be included on each request, i.e., name in full, identifying status, ward, previous transfusion history, and clinical diagnosis.

Upon receipt of the requisition forms, blood bank personnel should complete the second section of the forms, including the serial number of the blood unit being used, the group and Rh factor of the patient and the donor, the date of the crossmatch, and the signatures of the personnel performing and approving the crossmatch.

SF 518 should remain with the bottle at all times. Upon completion of the transfusion, one copy should be retained with the patient's records; the second copy should be returned to the blood bank with all post-transfusion data completed (third section of form) including the signature of the physician administering the bottle of blood. The third copy should be retained in the laboratory following the crossmatch. Any transfusion reaction should be noted on SF 518 and post-transfusion studies, if desired, should be requested on it.

Collection of Blood From the Patient

The blood bank technician, or person responsible, will collect blood from the patient only after positive identification by ward personnel, preferably the nurse or doctor, and a close check of the bed tag. In addition, the patient, if conscious, should always be asked his name. Information should be obtained concerning the estimated blood requirements of the patient if more than one unit is necessary and the approximate time it will be needed. Recipient blood sample should



CLINICAL RECORD

BLOOD TRANSFUSION

TRANSFUSION REQUISITION

BLOOD: ☒ WHOLE ☐ RED CELLS ☐ LIQUID PLASMA

AMOUNT: 500 ml

DATE REQUESTED: 20 Dec 1964

DATE AND HOUR WANTED: (STAT) 20 Dec 1964

PHYSICIAN'S SIGNATURE: M. L. Cowan

DIAGNOSIS: Mitral Stenosis

PREVIOUS TRANSFUSIONS: ☐ YES ☒ NO ☐ UNKNOWN

REACTIONS TO PREVIOUS TRANSFUSIONS: ☐ UNKNOWN ☒ NO ☐ YES (Type)

KNOWN IMMUNE ANTIBODY FORMATION: ☐ Rh ☐ OTHERS

IF PATIENT IS FEMALE, IS THERE HISTORY OF – ☐ ERYTHROBLASTOSIS ☐ STILLBIRTH ☐ MISCARRIAGE

REMARKS (Pertinent patient history)

I have taken a blood specimen on the below named patient, verified the name and checked the tube label.

D. M. Patrick
(Signature)

BLOOD GROUPING, COMPATIBILITY TESTS AND CERTIFICATION

TRANSFUSION NO: 04963

SERIAL NO. OF BLOOD: M-4130

SEROLOGY: Negative

DATE COLLECTED: 12/20/64

PATIENT: ABO GROUP [O] Rh TYPE Negative

DONOR: ABO GROUP [O] Rh TYPE Negative

COMPATIBILITY TESTS: ALS – Sal – AHG Negative
Visible Exam. Color Normal
PWY3 Appears Normal,
No suspicion of bacterial contamination

☐ RC/PS COMPATIBLE ☐ IN SALINE
☐ PC/RS COMPATIBLE ☐ IN HIGH PROTEIN
☐ INDIRECT ANTIGLOBULIN TEST ☐ RC/PS ☐ PC/RS

GROUP O BLOOD: (Universal donor) ☐ Rh NEGATIVE ☐ Rh POSITIVE

TITER, LESS THAN .... : ☐ SLIDE METHOD ☐ TEST TUBE METHOD ☐ SALINE ☐ HIGH PROTEIN

BLOOD GROUP SPECIFIC SUBSTANCES A AND B ADDED: ☐ YES ☐ NO

SIGNATURE OF TECHNICIAN: J E Campbell

DATE: 20 Dec. 1964

SIGNATURE OF CERTIFYING OFFICER: Joseph T. Brierre, Jr. LT(MC)USN

TRANSFUSION RECORD

DATE OF TRANSFUSION: 20 Dec 1964

AMOUNT GIVEN: 500 ml.

I have verified name, blood group and Rh type of recipient and donor (or donor No.) recorded on bottle labels and blood transfusion standard form and certify them to be correct.

I have further verified that the recipient is the same person named on bottle labels and transfusion forms.

REMARKS:

TRANSFUSION DIFFICULTIES

REACTION: ☒ NONE ☐ URTICARIA ☐ CHILL/FEVER
☐ OTHER (Specify)

If a hemolytic transfusion reaction is suspected, IMMEDIATELY –

1. Discontinue transfusion. Treat shock if present.
2. Notify Transfusion Service at once and send to laboratory.
   a. Unused blood and recipient set.
   b. Venous blood from patient—5 ml. in oxalate, 5 ml. dry.
   c. Immediate post-transfusion urine sample.
3. Save all post-transfusion urine.
4. Initiate intake-output record.

OTHER DIFFICULTIES (equipment, chills, etc.) ☐ YES (Specify) ☒ NO

DATE: 20 DEC 1964

SIGNATURE OF PHYSICIAN: M. L. Cowan

SIGNATURE:

PATIENT'S IDENTIFICATION (For typed or written entries give: Name—last, first, middle; grade; date; hospital or medical facility)

REGISTER NO.: J-41 96

WARD NO.: 3-C

Doe, John James
FN/N 3-C
USNH Bethesda, Md
12-20-64

BLOOD TRANSFUSION
Standard Form 518
(Revised)
518—110

Figure 11-14. SF 518, "Blood Transfusion."

be obtained in stoppered tubes which are identified by firmly attached labels bearing the recipient's full name, hospital number, and date.

ABO Grouping of Recipient

Grouping of the recipient's blood should be accomplished by two methods. The preferable approach is to test the red cells with anti-A and anti-B grouping sera meeting the National Institutes of Health (Division of Biologic Standards), standards and confirming the blood group by testing the serum with a pool of known group

A and known group B cells. An alternate acceptable method is to group the cells by two independent tests with two satisfactory sets of grouping sera of different lot numbers. The use of anti-A,B (O) serum is recommended as confirmation of all patients typed as group O to detect subgroups of A or B.

When a patient has received multiple transfusions of an ABO group different from his own, special attention should be given to possible errors in ABO (and Rh) grouping of the admixture of cells. Newborn recipients should be grouped with known anti-A- and anti-B sera. Serum grouping of the newborn may be misleading. The presence of auto-agglutinins or abnormalities serum protein may require saline suspension of the red cells, a 37° C. incubation temperature, or use of saline washed cells.

Rh Typing of Recipient

Normally, it is sufficient to test the recipient's blood with anti-D (Rh_o) serum only. This will determine whether the recipient shall receive Rh positive or Rh negative blood. To avoid having a recipient falsely labeled as Rh positive because of auto-agglutinins present in the serum, a control system employing recipient's cells in their own serum is highly recommended. The control also can be performed by retesting a saline suspension of the cells with an anti-D saline active serum. A recipient falsely identified as Rh positive may be immunized if he is transfused with Rh positive blood.

When women of childbearing age are candidates for transfusion, they should be further typed for C (rh′) and if possible c (hr′). If the recipient is found to be homozygous for C (rh′), every effort should be made to obtain donor blood which is also homozygous C (rh′). In order to best accomplish this, the age of all female candidates for transfusion should be entered in the remarks column, clinical record sections of SF 518.

Special Problems with Neonatal Blood

Blood grouping and Rh typing procedures performed on the cells of newborn infants may use venous blood, capillary blood, or umbilical cord blood. Since at birth the iso-agglutinins of the newborn's serum are derived from the mother, the serum grouping with known cells should be omitted. Milking the umbilical cord to obtain a sample of the child's cells should be avoided to prevent mixture with Wharton's jelly and subsequently false positive results.

Note. All samples of the recipient's blood should be stored in the refrigerator for 7 days after transfusion to permit a re-examination if necessary.

COMPATIBILITY TESTS—THE CROSSMATCH

[1] Unlike blood grouping tests, crossmatching tests are mixture of unknown sera and unknown cells. The heart of a blood bank or transfusion service is in its crossmatch bench. Every effort should be made to render crossmatching accurate. The tests used should be periodically re-examined. Changes should be unhesitatingly made, if, after careful consideration they are deemed advisable.

Prior to crossmatching it is necessary to obtain samples of blood from the intended recipient, the patient. Forms requesting blood samples from the recipient must include his full name, hospital number, ward, or room, sex, service and amount of blood desired. If possible, a history of previous transfusions and pregnancies should be obtained. Incomplete forms will not be accepted. The individual that draws the recipient's blood samples must take pains to be sure of the recipient's identity and the correctness of the labels on the samples.

Blood samples should be obtained in stoppered tubes identified with a firmly attached label bearing the recipient's full name, hospital number and date. The label must be attached to the tube before leaving the bedside and this tube, with the request slip, is sent to the blood bank.

On the request form and the labeled specimen, the identifying information must be reconfirmed by the technician in the blood bank before the sample is used for blood grouping and compatibility tests. In cases of discrepancies or doubt, another specimen must be obtained.

The recipient's ABO grouping is performed as previously described. It is sufficient to test the recipient's blood with anti-Rh_o (anti-D) serum only. This will determine whether the recipient should receive Rh positive or Rh negative blood.

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[1] From "Technical Methods and Procedures of the American Association of Blood Banks," American Association of Blood Banks.

All samples of the recipient's blood should be refrigerated for at least 4 days after transfusion to permit re-examination if necessary.

The older slide and test tube methods using saline suspended cells for crossmatching blood for transfusion were developed to demonstrate compatibility within the ABO groups only. These methods were rendered less useful by the discovery of the Rh-Hr blocking antibodies, since different conditions are required for the demonstration of these antibodies.

Anti-A and anti-B agglutinate specific cells regardless of the protein concentration of the serum. Anti-Rh, anti-Hr and other incomplete or blocking antibodies will not agglutinate specific bloods when the protein concentration in the serum mixture on the slide or in the test tube is below 5–10 percent. Agglutination is weak at 4–6 percent and optimal at 15–20 percent protein.

Consequently, it is necessary to employ routinely two crossmatch methods, one to prove compatibility within the ABO groups and a second to prove compatibility within other groups that form incomplete or blocking antibodies. The serum and saline methods are primarily for ABO groups, the high protein, Coombs (antiglobulin) and enzyme methods for other groups.

The crossmatch consists of two parts, the so-called major and minor sides. The major side is a mixture of donor's cells and the recipient's serum; the minor side is a mixture of recipient's cells and donor's serum. These are different parts of a crossmatch, not different methods or kinds. The major side must be compatible. The minor is sometimes incompatible, especially when O donor blood is given to recipients of other groups or an AB recipient received bloods of another group. Incompatibility in the minor side, however, must be explained or be understood before the unit of blood in question is transfused.

Guidelines in Summary[3]

1. Crossmatch:
    - *a*. Crossmatching shall be performed before administration of blood, except where delay may result in loss of life. The major crossmatch, the test for compatibility of donor cells and recipients serum, is required. The minor crossmatch, the test for compatibility of recipients cells and donor serum, is optional provided the donor's serum is adequately tested for regular and irregular antibodies.
    - *b*. The major crossmatch shall employ methods which optimally demonstrate the presence of complete and incomplete or blocking antibodies.
    - *c*. The major crossmatch shall include tests in (1) saline or serum and (2) antiglobulin (Coombs) systems. The saline or serum system shall utilize technics which are effective in the detection of complete and natural antibodies. The antiglobulin system shall utilize antihuman serum which meets the National Institutes of Health (Division of Biologic Standards) requirements and shall be used by a technic for which the serum was designed to be effective. As an acceptable alternate to (2) above, testing in an antiglobulin system may be performed as part of a screening examination of recipient serum against selected red blood cells, provided all aspects of the procedure are carefully controlled. The antigenic composition of the screening red cells shall be determined adequate in variety and strength for their intended purpose of detecting irregular antibodies, and additionally, shall meet National Institutes of Health (Division of Biologic Standards) minimum requirements for Reagent Red Blood Cells (Human). Antibodies against low frequency antigens will only be observed when the screening cells contain those antigens. When the screening test demonstrates an irregular antibody, the antiglobulin test must be part of all compatibility tests between donor cells and recipients serum. When the screening test does not demonstrate an irregular antibody, the

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[3] "Standards for a Blood Transfusion Service," American Association of Blood Banks.

compatibility procedure shall employ one of the test systems indicated in subheading *d* below. Screening tests shall be repeated as often as is indicated in item 2 below dealing with multiple transfusions.

d. In addition to the preceding methods, compatibility tests employing albumin, albumin serum or enzyme systems may be performed.

e. As complement is essential for the detection of some antibodies, crossmatching or screening procedures must employ fresh, noninactivated serum, not older than 48 hours, to insure its presence. Anticoagulants are anti-complementary, and therefore, plasma may not be used for the major crossmatch.

2. Multiple transfusions:

a. When transfusions are given over a period of days, a new sample of the recipient's blood should be obtained on the day of each transfusion so that irregular antibodies, which may have resulted from the previous transfusions, may be detected by the compatibility procedures. This practice also insures the presence of complement.

b. When a patient is to receive more than one unit of blood, it is not necessary to demonstrate compatibility between the various units of blood. Crossmatching for massive transfusions, including extracorporeal circulation, is adequately performed by testing cells from each donor and the recipient against a pool of donor sera (not more than five sera in each pool,) or by testing each donor's serum against Reagent Red Blood Cells (Human). Either technic must be followed by a major crossmatch.

3. Special problems of the new born:

Compatibility tests shall be performed before administering transfusions to the newborn. Since the antibodies present in the infant's serum are derived from the mother, it is preferable, particularly for exchange transfusions, to perform the crossmatch with the mother's serum and the donor's cells.

TESTING PROCEDURES

Since acceptable practices require two types of crossmatch for each transfusion, these are customarily performed simultaneously as outlined below. This procedure details a major crossmatch using saline suspended cells with a second major crossmatch with albumin added.

Major Crossmatch Technique

1. Place in a test tube rack two tubes for each donor unit being tested. These are labeled with the number of the crossmatch, and albumin and Coombs, respectively. Two additional tubes are added and labeled control and screen.
2. Add to each tube in this rack two drops of patients's serum.
3. Add:

a. To the albumin and Coombs tubes, one drop of a 5 to 8 percent suspension of donor cells, once washed in saline.

b. To the control tube, one drop of a 5 to 8 percent suspension of the patient's own cells. (This tube is to detect cold or other autohemagglutinins.)

c. To the screen tube, one drop of selected, pooled group O screen cells.

4. To each tube labeled albumin add two drops of 22 or 30 percent bovine albumin.
5. Centrifuge and read for agglutination or hemolysis.
6. Incubate for 30 to 60 minutes at 37° C.
7. Centrifuge all tubes for 30 seconds. Examine for hemolysis. Gently dislodge the button and examine for agglutination.

8. Wash the cells in the tubes marked Coombs, Control, and Screen, three to four times with sterile saline.
9. Decant completely and wipe away excess saline, and add two drops of antihuman (Coombs) serum, following manufacturer's instructions.
10. Centrifuge for 15 seconds and read immediately for agglutination.

Minor Crossmatch Technique

Note. The above procedure includes a screen for antibodies in the patient's serum supplanting the minor crossmatch. If this procedure is not followed, a minor crossmatch is mandatory.

1. Into a properly labeled test tube place one drop of a 5 percent suspension of patient's cells.
2. Add two drops of donor plasma from unit donor tubing segment.
3. Centrifuge and read for agglutination or hemolysis.
4. Incubate for 1 hour at 37° C. Centrifuge. Observe for hemolysis.
5. Wash three to four times with sterile saline and proceed with Coombs procedure as in step 8 under "Major Crossmatch Technique" above.

Enzyme Crossmatch Technique

In recent years some workers have advocated the use of various enzymes (papain, bromelin, ficin, etc.) in crossmatching procedures. The procedure shown in table 11-1 is exemplary of this alternate technique.

SUMMARY:

In summary, enzymes:

1. Produce crisp, tight agglutination which increases ease and speed of reading over methods employing colloid media.
2. Are more capable of detecting minimal amounts of antibody to some blood group systems than are colloid or antihuman globulin techniques.
3. Produce higher titers of antibodies to some blood group systems than colloid or antihuman globulin techniques.
4. Produce no prozones and diminish or abolish rouleaux formation.
5. Cause cells to absorb more antibody.
6. Cause cells to elute more antibody.
7. Give substantially higher titers of autoantibodies in acquired hemolytic anemia than with antihuman globulin techniques.
8. Do not usually improve the titers of saline agglutinins.
9. Inhibit or destroy antigen receptor sites in the MNS and Duffy systems.
10. Are more prone to pick up cold agglutinins than are colloid or antihuman globulin methods. If the direct method is used, this activity can be controlled by use of the patient's own cells and sera plus the enzyme, as in item 3*b*, "Major Crossmatch Technique."

Significance

When ABO group compatibility is proved, agglutination or hemolysis in the major side of the crossmatch indicates that the recipient, regardless of Rh type, may be immunized to specific Rh or Hr agglutinogens or other antigens on the donor cells. Further crossmatching with other donor bloods should be carried out unitl a blood is found which will not be agglutinated by the recipient's serum. Before testing with other donor bloods, the Rh type of the recipient should be carefully rechecked. Determination of rh′, rh″, and hr′ factors is additionally helpful.

Agglutination or hemolysis in the minor side of the crossmatch, when ABO compatibility is proved, may be due to Rh, Hr, or other antibodies in the serum of an immunized donor. When this occurs the donor's serum should be studied for specificity of the antibody.

Weak or doubtful incompatibility may be checked at refrigerator temperature. When there are no nonspecific cold agglutinins this check may provide a useful clue in identifying such antibodies as anti-M, anti-Le^a, and anti-P.

Table 11-1. Crossmatch Procedure

	TWO TUBE MAJOR		MINOR
	SALINE - (ALBUMIN) - COOMBS	ENZYME	SALINE
STEP 1	1. ADD 2 DROPS OF PATIENTS SERUM TO A SALINE WASHED BUTTON OF DONOR'S CELLS. 2. INCUBATE AT ROOM TEMPERATURE FOR 3-5 MINUTES. 3 CENTRIFUGE AND READ.	1. ADD 2 DROPS OF PATIENT'S SERUM TO A SALINE WASHED BUTTOM OF DONOR'S CELLS. 2. ADD 2 DROPS OF SPECTRAZYME.* MIX - INCUBATE AT ROOM TEMPERATURE FOR 3-5 MINUTES. 3. CENTRIFUGE AND READ.	1. ADD PATIENT'S CELLS TO 2 DROPS OF DONOR'S SERUM. 2. INCUBATE AT ROOM TEMPERATURE FOR 3-5 MINUTES. 3. CENTRIFUGE AND READ.
STEP 2	4. IF NEGATIVE - (MAY ADD 3 DROPS OF 22% OR 30% ALBUMIN) INCUBATE AT 37°C FOR 15-60 MINUTES. 5 CENTRIFUGE AND READ.	4. IF NEGATIVE. MAY INCUBATE AT 37°C FOR 10 MINUTES. 5. CENTRIFUGE AND READ.	NOTE: MAY PROCEED WITH STEPS 4-8 AS IN SALINE - (ALBUMIN) - COOMBS MAJOR.
STEP 3	6. IF NEGATIVE - WASH 3-4x WITH SALINE. COMPLETELY DECANT SALINE AFTER LAST WASH. 7. ADD 1-2 DROPS OF COOMBS SERUM - MIX. 8. CENTRIFUGE - SHAKE GENTLY, AND READ BOTH MACRO- AND MICROSCOPICALLY.		

NOTES:

1. CENTRIFUGE AT 1000 RPM FOR ONE (1) MINUTE OR SEROFUGE FOR FIFTEEN (15) SECONDS ONLY
2. IF POSITIVE MOST PROBABLY DUE TO A COLD AGGLUTININ SUCH AS ANTI-I OR ANTI H ETC.
3. IF POSITIVE, REPEAT USING PATIENT'S OWN CELLS AND SERUM PLUS SPECTRAZYME AS CONTROL

*STANDARD ITEM
FSN 6505-890-2053 BROMELIN,PAPAIN,FICIN ENZYME SOL.

DISCUSSION OF CROSSMATCHING

Nonspecific Agglutination

Mixtures of serum and red cells may show red cell agglutination not caused by specific antibodies in the serum but by non-specific effects. Such efforts must be appreciated lest they become a source of confusion. These effects are due to characteristics found in the serum. Precautions should be taken to remove even traces of such serum from red cells before the cells are mixed with other serum. A simple test for non-specific agglutination is to repeat the crossmatch using exactly the same technique but testing the individual's serum with his own cells. If this test is positive nonspecific agglutination is present.

Several types of nonspecific reaction are seen, those due to—rouleaux formation, auto-agglutinins, hyperproteinemia, or hyperglobulinemia effects and bacteriogenic changes.

Rouleaux Formation. Random human sera induce nonspecific agglomeration of red cells as shown by the red cells coming together, flat side to flat side, with variable numbers of cells in each mass and variable proportions of the red cells involved. The effect has been described as coin-roll appearance. In extreme instances, the characteristic coin-roll appearance is lost and the cell masses resemble the ragged masses seen in true agglutination. The coin-roll agglomeration is recognizable under the microscope if thin cell suspensions are employed. With very heavy cell suspensions rouleaux tendency expresses itself by a granular appearance in the field when the bulk of the cells is drained to the side.

Rouleaux formation is common under conditions which favor drying of cell-serum test mixtures. Thus material exposed to the open air may show rouleaux formation as time proceeds. Rouleaux formation may be seen when the room temperature is high and the humidity low, or when tests are carried out at altitudes high above sea level. Rouleaux formation is more commonly seen when the serum under test has a high original protein content, particularly when the globulin or fibrinogen level is high.

Rouleaux formation is of importance only if it interferes with interpretation of tests. The solution, therefore, is to avoid rouleaux formation whenever possible and, when it occurs to appraise accurately its extent and significance.

Simple measures to avoid drying are frequently adequate. Cell-serum mixtures in the tube are less prone to rouleaux than on the slide and may be additionally protected by corking. Mixtures on the slide should be read promptly or may be covered with a cover slip (well-slides are preferable if a cover slip is used) or may be placed within a suitable container such as a Petri dish and additionally protected by placing saline within the same container but separate from the test mixture. Rouleaux formation may be prevented by addition of saline to the cell-serum mixture. As might be expected, tests involving saline suspensions of cells are less likely to show rouleaux formation than tests in which the cells have been suspended in the individual's own serum or plasma. Rouleaux formation is reversible by mechanical agitation, e.g., stirring with an applicator. However, if this technique is to be employed, it is important to examine the mixture very promptly after agitation, for the disassociation is transient.

In all cases it is advisable to establish that the agglomeration is due to nonspecific effects and not to true agglutination by repeating the crossmatch with the use of antihuman serum (indirect test); if the indirect test with antihuman serum is negative, the interpretation of rouleaux is supported. A positive indirect antiglobulin test would indicate that a true antigen-antibody reaction is present (perhaps in addition to rouleaux formation).

Auto-agglutinins, Cold Agglutinins and Warm Agglutinins. An antibody that agglutinates cells from its own blood is an auto-agglutinin. Most auto-agglutinins, but not all, clump cells most readily in the cold and, hence, they are often called cold agglutinins. When an agglutinin clumps cells most readily in a warm atmosphere, e.g., at 37° C., it is known as a warm agglutinin. Cold and warm agglutinins are usually panagglutinins, because they agglutinate all cells.

The proof of the presence of a cold agglutinin is—

1. Reactivity in low temperature tests with cells from the original specimen.
2. Reactivity in low temperature tests with cells from all other specimens.

3. **Lack of or weaker reaction when the tests are repeated at 37° C. and disappearance of agglutination upon change from low temperature to 37° C.**

The strength of cold agglutinins varies markedly as judged by titrations performed at low temperatures or by studies of the temperature range over which the cold agglutinin effect is seen. With very delicate tests traces of cold agglutinin can be demonstrated in most bloods. However, the term is generally restricted to instances in which the titer is high and the range of temperature activity extends to room temperature or even higher. One should keep in mind that centrifugation may cool a previously warmed serum-cell mixture and thus bring about cold agglutination.

Depending on the quantity of cold agglutinin in the original blood specimen and the temperature at which cells and serum are separated, a cold agglutinin may be found on cells or in serum, or both. In an extreme instance a specimen may appear to be group AB regardless of the true blood group. The serum from such a specimen will agglutinate both A cells and B cells producing the paradox of group AB cells in group O serum. Red cells with an attached cold agglutinin ordinarily release them if the temperature is raised to 37° C.

Washing the cells with saline solution at 37° C., centrifuging at this temperature, and resuspending the cells in fresh saline usually eliminates cold agglutinins and permits accurate determination of the blood group.

A serum containing a cold agglutinin when crossmatched at 37° C., will show no cold agglutinin effects. Reading of such tests should be made promptly before the mixture cools.

It is permissible and safe to transfuse in the presence of cold agglutinins if the blood grouping and crossmatching procedures are otherwise satisfactory when tests are performed at 37° C. Cold agglutinins may intrude into tests with antihuman serum and cause positive reactions unless they are eliminated by washing with warm saline.

Hyperproteinemia and Hyperglobulinemia Effects. Occasional human sera may have a very high total protein or globulin content: (e.g., in multiple myeloma). Such unusually high protein levels are likely to be associated with extreme tendency to rouleaux formation and may cause agglutination of all cells tested, including those from the original blood specimen. The determination of the proper blood group may require several preliminary washings of the red cells with saline at 37° C. Proper determination of the serum may require tests at 37° C., together with the addition of noninterfering diluents such as 0.5 ml. saline to mixtures in saline tube tests, or 0.5 ml. of 15 percent albumin in high protein tube tests. Crossmatching tests involving such specimens may require similar precautions. The indirect antiglobulin test, if negative, also establishes compatibility.

Precautions in the Use of Testing Sera

1. If frozen, thaw completely and mix before use.
2. Thaw at room temperature, some antibodies are thermolabile.
3. Watch the expiration date for outdating and do not use beyond the expiration date.
4. *Performance:* It is essential for the technician to have complete confidence in the sera used. Such confidence is made possible by consistency in a serum's agglutinating ability, or, in the case of sera infrequently used, by optimal agglutination of suitable control cells.
5. Refrigerate when not in use.
6. Follow the manufacturer's directions. Before using serum from a new manufacture, it is *imperative* to read the directions. Even serum similarly labeled or similar in appearance require different conditions.
7. Examine for bacterial contamination. The presence of bacteria or products of bacterial growth may have a marked effect upon reactions of red cells and sera. One effect may be to reduce reactivity. For example, a contaminated serum may show a marked drop in agglutinating ability. More commonly observed, however, is the appearance of nonspecific reactions (false positives). Thus, contaminated group O cells may be apparently aglutinated by anti-A or anti-B blood grouping sera. Conversely, a contaminated serum may agglutinate

5a23

both positive and negative cells. Similar effects may be seen if saline, albumin, or other reagents, or glassware are contaminated.

Awareness of these possibilities provides the basis for making every reasonable effort to achieve and maintain sterility in blood bank work. Some diagnostic testing sera have preservatives added which reduce but do not eliminate the risks. Care to avoid entrance of bacteria reduces the hazard. Careful attention to storing blood and sera in the refrigerator, when not in use, lessens the growth of any bacteria which may be present. It is particularly important to close containers promptly after use and to hold exposure to the open air to a minimum. Unexpected bizarre reactions which cannot be duplicated when fresh material and clean, dry glassware are used are suggestive of bacterial or chemical contamination. Trace amounts of detergent remaining in inadequately rinsed glassware can cause serious trouble. Follow the manfacturer's directions.

Additional Considerations

In order to adequately perform and understand the crossmatch procedure it is necessary that certain precautions be observed. These are indicated below.

1. All equipment employed in crossmatching procedures must be correctly labeled.
2. A useful simple test for nonspecific agglutination is to repeat the crossmatch using exactly the same technique but testing the patient's serum with his own cells.
3. When performing the antihuman globulin crossmatch it must be realized that the activity of a particular antihuman serum is most likely to be at its best if the manufacturer's instructions are closely followed.
4. Freshly prepared cell suspensions should always be used in crossmatching.
5. In the antihuman globulin crossmatch, excessive washing of the cells may remove the coating antibodies if they are present. The washing must be sufficient, however, to remove all protein except that which is immunologically coating the cells.
6. When a series of transfusions are to be given over a period of days, it is essential that a new sample of the recipient's blood be obtained on the day of each transfusion so that atypical agglutinins, which may have resulted from the previous transfusions, may be detected by the crossmatch procedures.
7. Causes of false compatible crossmatches include—
   - *a.* Manipulations in which the donor's or recipient's cells and serum are inadvertently combined.
   - *b.* The presence of a potent isohemolysin which destroys the agglutinates before they may be observed. If hemolysis leads to confusion in proper interpretation of the crossmatch, the patient's and donor's serum should be inactivated at 56° C. for 30 minutes and the crossmatched reperformed.
   - *c.* Excessive shaking of the tubes prior to reading, thus breaking up weak agglutinates.
   - *d.* Weak antihuman serum in the antihuman globulin crossmatch.
   - *e.* Insufficient or excessive cell washing in the antihuman globulin crossmatch.
   - *f.* Improper cell concentrations.
   - *g.* Improper incubation periods.
   - *h.* Cells improperly prepared or inaccurately designated.
   - *i.* Hemolyzed cells.
   - *j.* Addition of saline at improper time.
   - *k.* Blood specimens which have been retained too long after being drawn.
   - *l.* Deteriorated or contaminated patient's and/or donor's serum.
   - *m.* Excessively heavy cell suspension.
   - *n.* Outdated or contaminated antihuman serum and/or bovine albumin.
8. Causes of false incompatible crossmatches include—
   - *a.* Rouleaux formation.
   - *b.* Drying of serum-cell mixtures.
   - *c.* High titers of cold hemagglutinins.
   - *d.* Sedimentation of cells due to insufficient mixing.
   - *e.* Cell hemolysis due to rough handling of the blood prior to the cross match.

f. Excessive centrifugation.

g. Using antihuman serum containing other than antihuman globulin antibodies.

h. Nonspecific agglutination due to chemical contamination of the glassware or bacterial contamination of the serum or cells.

i. Nonspecific agglutinins.

j. Misinterpretation of the appearance of the cell mass.

k. In the antihuman globulin crossmatch, insufficient incubation time following the addition of antihuman serum.

l. Coagulation due to excessive heating.

ISSUING OF BLOOD

SF 518, in triplicate, must be completed by the blood bank technician for each unit of blood to be issued for transfusion. The completed forms should be securely attached to the proper bottle of blood by means of a rubber band. The blood, then, will be kept in the blood bank refrigerator until it is ready for use by the physician. At that time, he will request one unit unless two or more bottles are to be used simultaneously or in rapid sequence. Under no circumstances should blood be permitted to remain on the wards, unused, at room temperature. The blood bank should accept no responsibility for returned blood which has been out of its control. Blood should never be warmed artificially before transfusion.

When the transfusion has been accomplished, the SFs 518 are to be completed by the physician or nurse and one copy returned to the laboratory the same day. Upon the receipt of this copy by the laboratory the third copy, which was retained when issuing the unit of blood, may be discarded. A second copy is retained on the ward and made a part of the patient's permanent record. Appropriate notes on all reactions or other necessary information should be recorded on the spaces provided.

No unit of blood should be returned to the blood bank for reissue if the bottle has been entered for the purpose of transfusing a patient. In such an instance there is the possibility that contamination of the blood may have taken place.

TRANSFUSION REACTIONS AND COMPLICATIONS

The transfusion of whole blood or its components is a safe procedure only when every precaution has been taken to safeguard the operation. There are many complications in blood transfusion which may cause untoward or even fatal reactions. These undesirable effects may be avoided when there is a full awareness of the causative factors. Some of the more important transfusion complications are discussed below.

Hemolytic Transfusion Reactions

Every effort must be made to prevent hemolytic reactions due to the transfusion of incompatible red cells. Very careful attention must be paid to the techniques of blood grouping, Rh typing, and crossmatching. The technician must recognize the possibilities of immunization that exist in individuals requiring repeated transfusions and in females who have had one or more pregnancies.

Blood samples withdrawn from patients for grouping, typing, and crossmatching must not be mixed, and the blood crossmatched for one patient must not be transfused into another (a frequent cause of hemolytic reactions). In recent years blood has become more available for the treatment of individuals and there is a tendency for some persons who transfuse blood to regard it lightly. Rigid controls must be established to insure that the recipient will be properly identified and that he will receive the blood which has been crossmatched with the sample of blood taken from him.

The hemolytic reaction occurs as a result of donor erythrocytes being destroyed by antibodies present in the plasma of the recipient. This type of reaction is accompanied by a high mortality rate and, for this reason, it is mandatory that the reaction be detected as early as possible in order to prevent further damage. The clinical signs of a hemolytic reaction may vary greatly among patients, probably depending upon the antibody concentration in the recipient's plasma. The reaction may occur after the transfusion of as little as 25-50 ml. of blood. On occasion an individual may receive 500 ml. of blood and shown no immediate signs of the hemolytic reaction to come. As a rule, however, after the administration of 25-200 ml. of blood, the patient will suffer a severe chill, pain in the lumbar region, nausea, vomiting, and a rapid elevation of temperature. It is important, therefore, that a competent individual observe the recipient during the administration of the first 25-100 ml. of blood in order to anticipate a reaction and stop the transfusion at the earliest possible time. There may be the appearance of substernal pain or involuntary urination or defecation. The blood pressure may increase early but usually drops, and with the associated rapid pulse rate, will indicate that the patient has passed into a condition of shock.

This initial reaction is rarely fatal. Usually the individual will appear to improve within a few hours. The first specimens passed after a hemolytic reaction will be dark reddish-brown in color and contain protein, red cells, pigment casts, and will give a positive bensidine test for blood.

Following recovery from the initial reaction, the patient usually exhibits no further symptoms except a mild transient bilirubinemia or mild jaundice. In other individuals there may be a decreased urine output with a significant but transitory nitrogen retention followed by a period of increased urine excretion and recovery. Other patients may experience persistent oliguria, often developing into complete anuria with greatly increased nitrogen retention and the development of uremia. Some of these patients may have a diuresis about the 10th day and recover. Others in whom the anuria persists and diuresis does not occur, die as a result of renal failure.

It is important to realize that there are many conditions requiring transfusion that may bring about the same renal picture that occurs following a transfusion reaction. Individuals suffering from deep wounds or burns associated with deep shock may develop the kidney damage similar to that found in individuals who have been given incompatible blood.

Note. AS SOON AS A HEMOLYTIC TRANSFUSION REACTION IS SUSPECTED, THE ADMINISTRATION OF BLOOD SHOULD BE DISCONTINUED *IMMEDIATELY*.

Pyrogenic Reactions

The term pryrogen refers to a substance which is fever producing. Pryogen reactions may occur as a result of the introduction in the blood stream of foreign materials such as inspissated protein, dried blood, viable or nonviable organisms, or the by-products of the growth of certain bacteria. Pyrogenic reactions may occur with any solution which is administered intravenously and symptoms will vary with the amount of pyrogenic material injected.

Pyrogens are frequently found in surface waters and are not removed by filtration or chemical sterilization. Proper distillation of water will often render it nonpyrogenic. If sterile water becomes contaminated with pyrogen producing bacteria, it will, in the matter of 4 hours or more, become pyrogenic when injected into man.

Pyrogenic reactions may vary in severity from a mild chilliness with no temperature increase to severe chills and a rise in temperature to 103° F. and higher. This type of reaction may occur at any time during or after the administration of blood but occurs most frequently within 30-60 minutes after the transfusion has been completed. The patient will usually appear to be flushed, may become nauseated and vomit, and usually suffers a severe headache with muscle aches and pains. The temperature, as a rule, returns to the pretransfusion level within 4 hours.

There is no clear-cut way to differentiate the onset of a serious hemolytic reaction due to incompatible blood from the initial symptoms of a pyrogenic reaction. For this reason, the administration of blood must be discontinued immediately with the onset of chills. Pyrogenic reactions may be fatal if the quantity of pyrogenic material injected is great.

Contaminated Blood

Blood may become contaminated with bacteria and fungi from two sources. These are (1) failure to adequately prepare the venipuncture site on the donor's arm or contamination of the needle during bleeding, and (2) failure to have sterile equipment, needles, tubing, and contents of blood containers.

Most of the organisms which contaminate whole blood are gram-negative, coliform, or pseudomonas types. Gram-positive organisms more often contaminate plasma. Organisms which grow in blood produce pyrogenic substances which, in sufficient quantity, may cause extremely severe reactions and even death. It is unfortunate that organisms of the pseudomonas type grow well at icebox temperatures. While it is important to keep blood in storage at 4°-6° C., it can be seen that bacterial growth is not prevented in every instance.

Lightly contaminated blood may give reactions indistinguishable from the common pyrogenic reactions. These include chills and fever. If blood is grossly contaminated, the symptoms are chills, high fever, generalized aches and pains and rapid onset of hypotention and shock. The skin exhibits a marked erythema which is in contrast to the pale, cold skin encountered in hematogenic shock.

Allergic Reactions

Allergic reactions may take place following the transfusion of whole blood, plasma, or cells. This type of reaction is most frequently characterized by the appearance of urticaria but occasionally there may be a diffuse rash or angioneurotic edema which may involve the larynx. Less frequently, asthmatic seizures may complicate the transfusion of blood. Allergic reactions are probably due to a response by the recipient to allergenic substances in the donor blood. There is also a possibility that some recipients may be passively sensitized by administering plasma donated by an allergic individual. An allergic reaction to blood transfusion usually occurs during the administration of blood or within 1 or 2 hours thereafter. The typical symptoms are itching of the skin, development of hives, or a diffuse rash. On occasion, there will be edema of the face or lips, or within the oral cavity. Sometimes typical wheezing and difficulty in expiration are observed.

Circulatory Overload

When the patient has a reduced blood volume, he may be transfused very rapidly. When blood is given to an individual in severe shock, it is not only permissible but advisable to administer it at a rate of 50-75 ml. per minute or faster.

If shock is not present, the transfusion of blood may be started at a rate of approximately 2 ml. per minute for the first 20 minutes. The rate can then be increased so that the procedure may be finished in approximately 45 minutes. When this procedure is followed, most hemolytic reactions will be detected before more than 100 ml. of blood have been administered and the transfusion can be discontinued. Also, when employing an initial slow rate of flow, it will be simple to judge the tolerance of the patient for the quantity of blood to be given.

As a rule, blood is transfused into patients to increase (1) the blood volume in shock or (2) the oxygen-carrying capacity of the blood. In people who are elderly or who have cardiac conditions, a state of anemia is sometimes present. In such individuals sudden changes in blood volume are not well tolerated. Cardiac muscle which is weakened cannot be expected to support the sudden increase in blood volume which always results when blood or plasma is injected. These patients may rapidly develop myocardial insufficiency and pulmonary edema. In these individuals, red cell suspensions, from which the plasma has been removed will give rise to fewer overload reactions than whole blood.

It is extremely important that the characteristics of the overload reaction be recognized. Circulatory overload usually occurs during the administration of blood or shortly after the completion of the transfusion. It is characterized by a sudden difficulty in breathing, cyanosis, cough, and frothy sputum, often blood tinged. Stethoscopic examination of the lungs will reveal moist rales throughout the chest. The patients may die in a matter of a few minutes unless prompt corrective measures are taken.

Embolic Reactions

Blood and plasma must be filtered immediately prior to administration in order to remove any particles which might be present. This procedure is so widely practiced that there are very few reports of untoward transfusion reactions following administration without filtration of the blood.

Air emboli may bring about fatal results. There is a possibility that air will be introduced into the circulation if the recipient tubing is not completely filled with blood before the venipuncture is accomplished. More often air emboli result when blood is forced into the patient under pressure. The use of pressure is justified in many instances but must never be resorted to unless a competent responsible individual is present. The use of plastic blood containers avoids the possibility of air embolism.

Transmission of Disease

Of the diseases which may be passed from individual to individual by blood transfusion, the most important are infectious hepatitis, malaria, and syphilis. Infectious hepatitis may be passed from one person to another by blood transfusion and contaminated needles, as well as by transmission of droplets through the mucous membranes. Recent evidence suggests that the blood may contain the causative organism for many months or even years. One type of hepatitis known as homologous serum jaundice apparently occurs only after the transfusion of human blood. It appears to have an extended incubation period. The appearance of symptoms occurs from 60–180 days after the administration of blood. The causative agent of this disease has not been identified. It appears to resist refrigerator storage, the action of certain preservatives, and the application of moderate amounts of heat over a short period of time. So far no effective means of removing the causative agent from whole blood has been developed. The agent is passed to recipients in spite of careful questioning of donors regarding the occurrence of hepatitis. The exclusion of any donors who give a history of jaundice due to causes other than obstruction of the bile tract is recommended. It must be realized, however, that hepatitis following transfusion is always a possibility.

Malaria has been transmitted by transfusion of blood. The parasites remain viable in refrigerated blood for at least a few days. The period of storage sufficient to insure death of the organisms is not known. Syphilis may also be transmitted through the administration of blood. This transmission may occur in the early stages before the serologic tests become positive. Evidence indicates that refrigeration at 4° to 5° C. for 1 to 3 days will render the treponemes incapable of producing the disease.

Many other diseases may be transmitted if blood is collected during the phase of the condition in which the causative organism is present in the bloodstream. This includes diseases characterized by bacteremia and most of the rickettsial and virus diseases, such as relapsing fever, etc. The possibility of the transfer of disease must be kept in mind during the screening of blood donors. Whenever such a disease or condition occurs in the donor within a short time following the donation of blood, every effort must be made to locate and discard his blood. If it has already been administered, the physician of the recipient should be notified of the possibility that a complication may develop.

LABORATORY INVESTIGATION OF TRANSFUSION REACTIONS

If a hemolytic transfusion reaction is suspected, certain investigative procedures should be carried out. A recommended plan of investigation is discussed below.

1. The transfusion should be stopped immediately if signs of a reaction appear.
2. The laboratory officer or physician in charge of the blood bank should be notified immediately.
3. The blood container with any unused blood and the transfusion set should be sent to the laboratory. The label on the container of blood should be re-examined to be sure that there has been no mistaken identity of the patient.
4. As soon as possible after the onset of symptoms, 10 ml. of venous blood should be drawn from the patient. The blood should be placed in a clean, dry test tube avoiding hemolysis and sent to the laboratory. This blood must be tested for bilirubinemia and hemoglobinemia. Serum bilirubin should be checked again at the end of 5 hours and 24 hours.
5. All urine specimens collected during the first 24 hours following a transfusion reaction should be sent to the laboratory and tested for hemoglobinuria.
6. The blood remaining in the bottle should be cultured at 4°–6° C. and 30°–32° C.

A stained smear of donor blood should be examined for bacteria. The toxins produced by certain bacteria, particularly saprophytic bacilli that do not grow readily, if at all, at 37° C., do not usually promote hemolysis of blood cells but produce shock and severe clinical manifestations.

7. The blood group and Rh type of the patient should be redetermined from the pre-transfusion specimens (saved in the blood bank refrigerator).
8. Similarly, the blood group and Rh type should be redetermined on the blood in the pilot tube of the donor and in the returned container of blood.
9. The major crossmatches should be repeated and an indirect antiglobulin (Coombs) test should be included.
10. A direct antiglobulin (Coombs) test should be performed on the pre- and post-transfusion patient specimens of blood.
11. Both copies of the SF 518 should be returned to the laboratory with the specimens in order that the laboratory date may be entered on the sheets.

TRANSFUSION SERVICE RECORDS

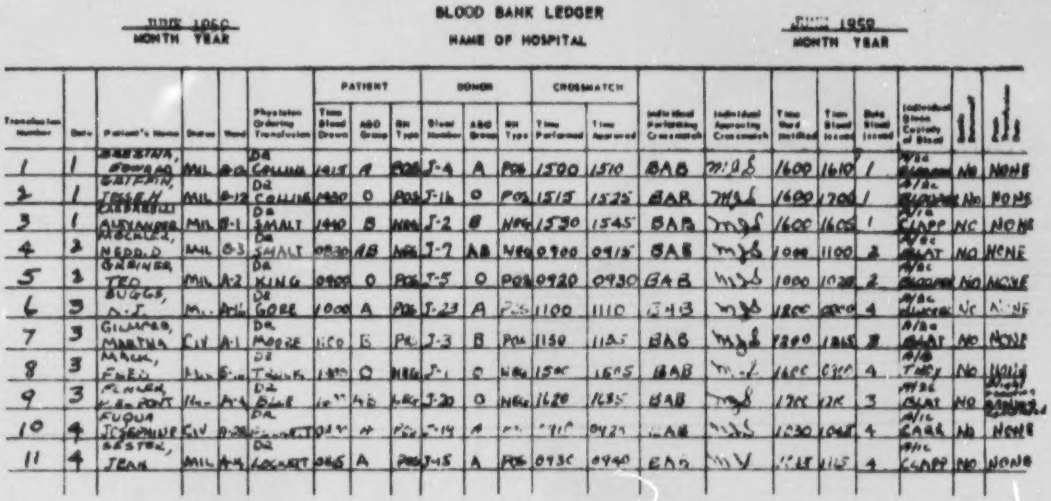
The maintenance of adequate records is essential for an approved blood transfusion service. Each blood transfusion service must develop a system of record keeping which best serves its needs. Samples of records concerning the various phases of the transfusion service are presented below. Records pertinent to the operation of a blood donor center are included in chapter 10.

Blood Bank Ledger

Blood bank personnel should assign a number obtained from the Blood Bank Ledger (fig. 11-15) to the recipient before a specimen of blood is obtained. This number, the date, the recipient's name and identifying status, the ward and name of the physician requesting the transfusion should then be recorded in the Blood Bank Ledger. A separate transfusion number is assigned to each transfusion of 500 ml. or less.

Upon completing a crossmatch, the blood bank technician should complete those sections of the ledger which include the blood group and Rh factor of the patient and the donor, the bottle number, and the time the crossmatch was performed. He should then enter his initials in the appropriate space. The individual approving the crossmatch should likewise enter his initials and time of approval.

The remainder of the ledger should be completed when the patient's ward or operating room



JUNE 1959
MONTH YEAR

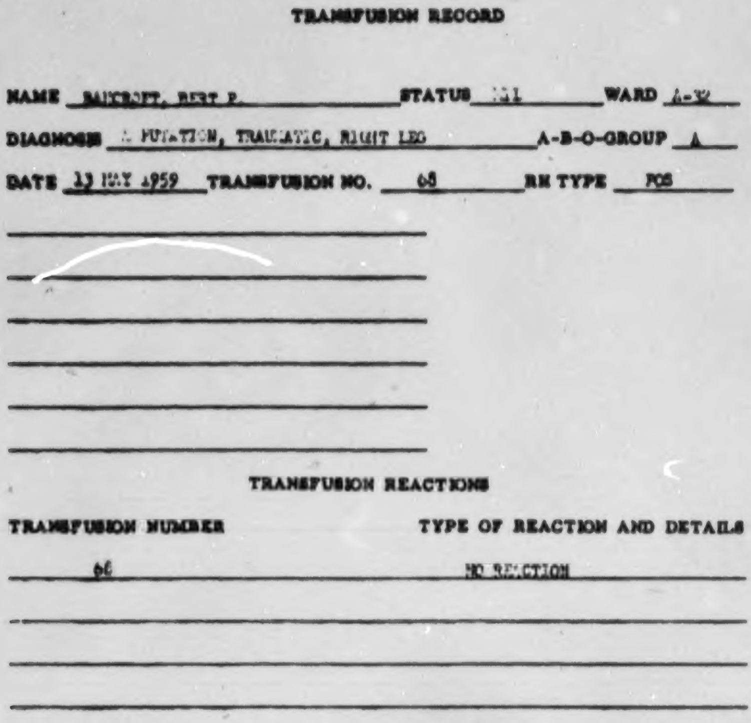
BLOOD BANK LEDGER
NAME OF HOSPITAL

JUNE 1959
MONTH YEAR

Transfusion Number	Date	Patient's Name	Status	Ward	Physician Ordering Transfusion	PATIENT			DONOR			CROSSMATCH		Individual Performing Crossmatch	Individual Approving Crossmatch	Time Ward Notified	Time Blood Issued	Date Blood Issued	Individual Given Custody of Blood	[illegible]	[illegible]
						Time Blood Drawn	ABO Group	Rh Type	Blood Number	ABO Group	Rh Type	Time Performed	Time Approved								
1	1	BARSTAR, EDWARD	MIL	B-[illegible]	DR COLLINS	1415	A	POS	J-4	A	POS	1500	1510	BAB	MJS	1600	1610	1	A/3C [illegible]	NO	NONE
2	1	GRIFFIN, JESSE H	MIL	B-12	DR COLLINS	1420	O	POS	J-16	O	POS	1515	1525	BAB	MJS	1600	1700	1	A/3C [illegible]	NO	NONE
3	1	[illegible] ALEXANDER	MIL	B-1	DR SMALT	1440	B	NEG	J-2	B	NEG	1530	1545	BAB	MJS	1600	1605	1	A/C CLAPP	NO	NONE
4	2	MICHALS, NEDD D	MIL	B-3	DR SMALT	0830	AB	NEG	J-7	AB	NEG	0900	0915	BAB	MJS	1000	1100	2	A/3C BLAT	NO	NONE
5	2	GREINER, TED	MIL	A-2	DR KING	0905	O	POS	T-5	O	POS	0920	0930	BAB	MJS	1000	1020	2	A/3C [illegible]	NO	NONE
6	3	BUGGS, A. J.	MIL	A-4	DR GORE	1000	A	POS	J-23	A	POS	1100	1110	BAB	MJS	1200	[illegible]	4	A/3C [illegible]	NO	NONE
7	3	GILMORE, MARTHA	CIV	A-1	DR MOORE	1100	B	POS	J-3	B	POS	1130	1145	BAB	MJS	1200	1215	3	A/3C BLAT	NO	NONE
8	3	MACK, FRED	MIL	B-[illegible]	DR [illegible]	1500	O	NEG	J-1	O	NEG	1500	1505	BAB	MJS	1600	[illegible]	4	A/3 TRACY	NO	NONE
9	3	[illegible], [illegible]	MIL	A-4	DR BLUE	[illegible]	AB	NEG	J-20	O	NEG	1620	1635	BAB	MJS	1700	1715	3	A/3C BLAT	NO	[illegible]
10	4	FUQUA JOSEPHINE	CIV	B-3	DR [illegible]	[illegible]	A	POS	J-14	A	POS	[illegible]	0925	BAB	MJS	1030	1045	4	A/1C CARR	NO	NONE
11	4	BESTER, JEAN	MIL	A-4	DR LOCKETT	0915	A	POS	J-15	A	POS	0930	0940	BAB	MJV	1115	1115	4	A/1C CLAPP	NO	NONE

Figure 11-15. Blood bank ledger.

513



TRANSFUSION RECORD

NAME BANCROFT, BERT P. STATUS [illegible] WARD A-32

DIAGNOSIS AMPUTATION, TRAUMATIC, RIGHT LEG A-B-O-GROUP A

DATE 13 MAY 1959 TRANSFUSION NO. 68 RH TYPE POS

TRANSFUSION REACTIONS

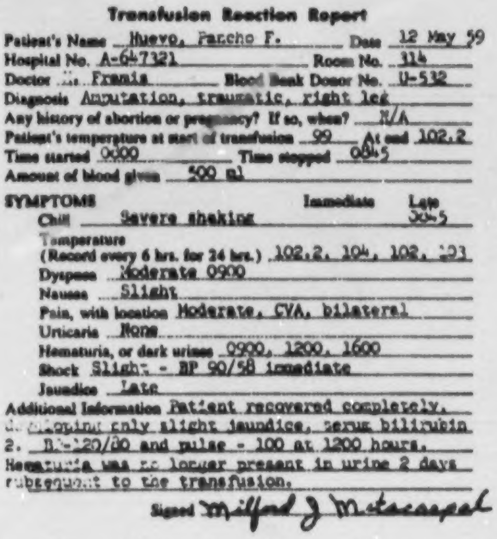
TRANSFUSION NUMBER	TYPE OF REACTION AND DETAILS
68	NO REACTION

Figure 11-16. Transfusion record form.

is notified, when the blood is received by the ward or operating room, and finally when the empty bottle is returned to the blood bank. The completed ledger should be a permanent record of the operations performed by the blood bank in preparing blood for transfusion. It is invaluable as such and should be kept accurate and neat at all times.

Transfusion Record

This is a record which may be kept on all transfused individuals. It includes the name, status, ward, clinical diagnosis, and ABO group and Rh type. The date and transfusion numbers of blood actually administered to the patient are recorded on this card. Any adverse reaction is noted on the bottom third of the card. Its assistance in rendering readily accessible information in the ABO groups and Rh types of currently hospitalized patients and on the number of transfusions previously given can be of great aid to the blood bank. A typical transfusion record is illustrated in figure 11-16. More elaborate transfusion reaction report forms are illustrated in figures 11-17 and 11-18.



Transfusion Reaction Report

Patient's Name Huevo, Pancho F. Date 12 May 59

Hospital No. A-647321 Room No. 314

Doctor Franis Blood Bank Donor No. U-532

Diagnosis Amputation, traumatic, right leg

Any history of abortion or pregnancy? If so, when? N/A

Patient's temperature at start of transfusion 99 At end 102.2

Time started 0900 Time stopped 0845

Amount of blood given 500 ml

SYMPTOMS Immediate Late

Chill Severe shaking 0845

Temperature (Record every 6 hrs. for 24 hrs.) 102.2, 104, 102, 101

Dyspnea Moderate 0900

Nausea Slight

Pain, with location Moderate, CVA, bilateral

Urticaria None

Hematuria, or dark urines 0900, 1200, 1600

Shock Slight - BP 90/58 immediate

Jaundice Late

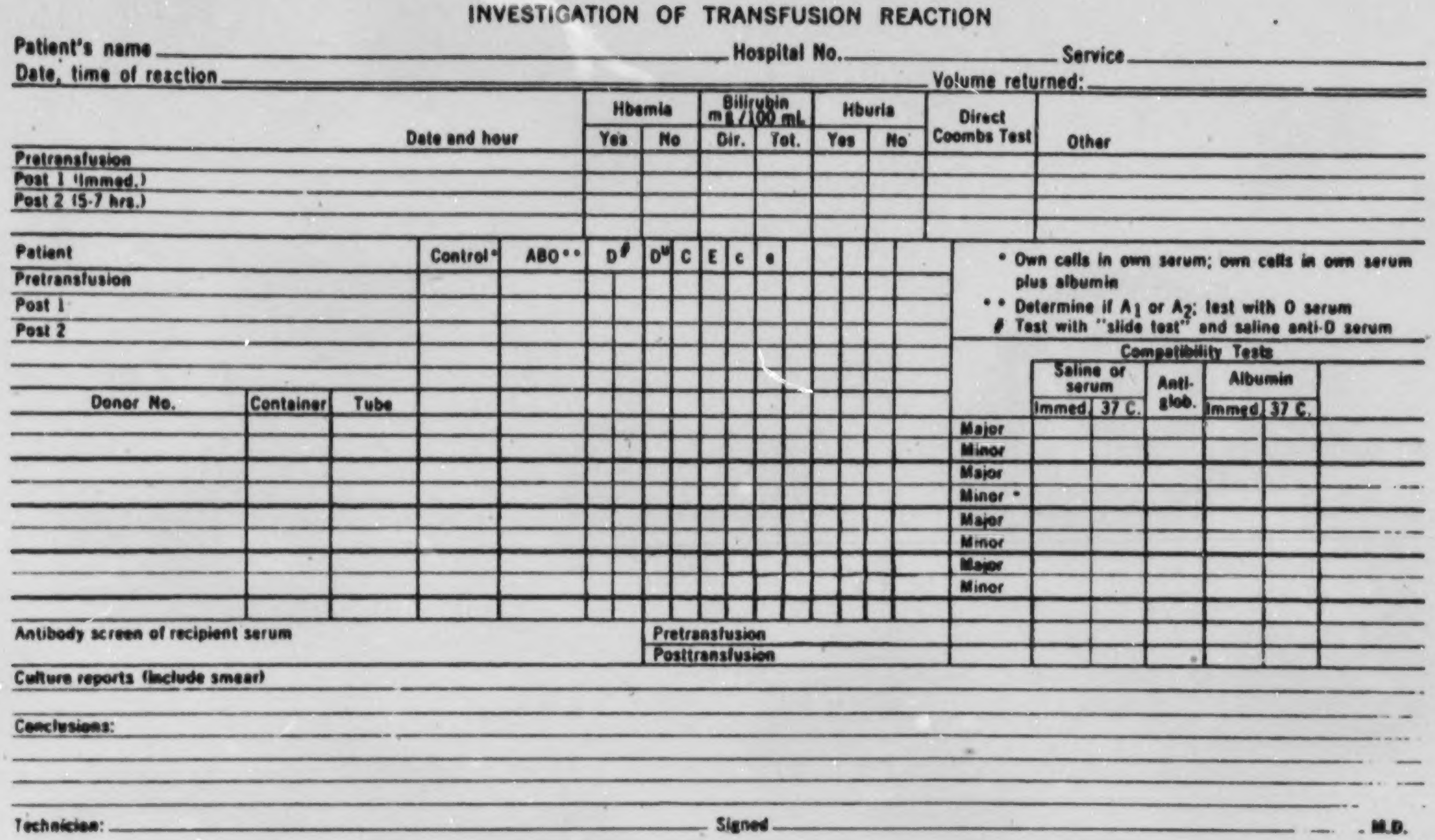
Additional Information Patient recovered completely, developing only slight jaundice, serum bilirubin 2. BP-120/80 and pulse - 100 at 1200 hours. Hematuria was no longer present in urine 2 days subsequent to the transfusion.

Signed Milford J. Metacarpal

Figure 11-17. Transfusion reaction report.

Identification Tags

The proper identification of a patient to be transfused is of utmost importance. It limits the element of human error and greatly reduces the



INVESTIGATION OF TRANSFUSION REACTION

Patient's name ______ Hospital No. ______ Service ______

Date, time of reaction ______ Volume returned: ______

	Date and hour	Hbemia Yes	Hbemia No	Bilirubin mg/100 mL Dir.	Bilirubin mg/100 mL Tot.	Hburia Yes	Hburia No	Direct Coombs Test	Other
Pretransfusion									
Post 1 (Immed.)									
Post 2 (5-7 hrs.)									

Patient	Control*	ABO**	D#	D^u	C	E	c	e
Pretransfusion								
Post 1								
Post 2								

Donor No.	Container	Tube

* Own cells in own serum; own cells in own serum plus albumin

** Determine if A_1 or A_2; test with 0 serum

# Test with "slide test" and saline anti-D serum

Compatibility Tests

	Saline or serum Immed.	Saline or serum 37 C.	Anti-glob.	Albumin Immed.	Albumin 37 C.
Major					
Minor					
Major					
Minor *					
Major					
Minor					
Major					
Minor					

Antibody screen of recipient serum

Pretransfusion

Posttransfusion

Culture reports (include smear)

Conclusions:

Technician: ______ Signed ______ M.D.

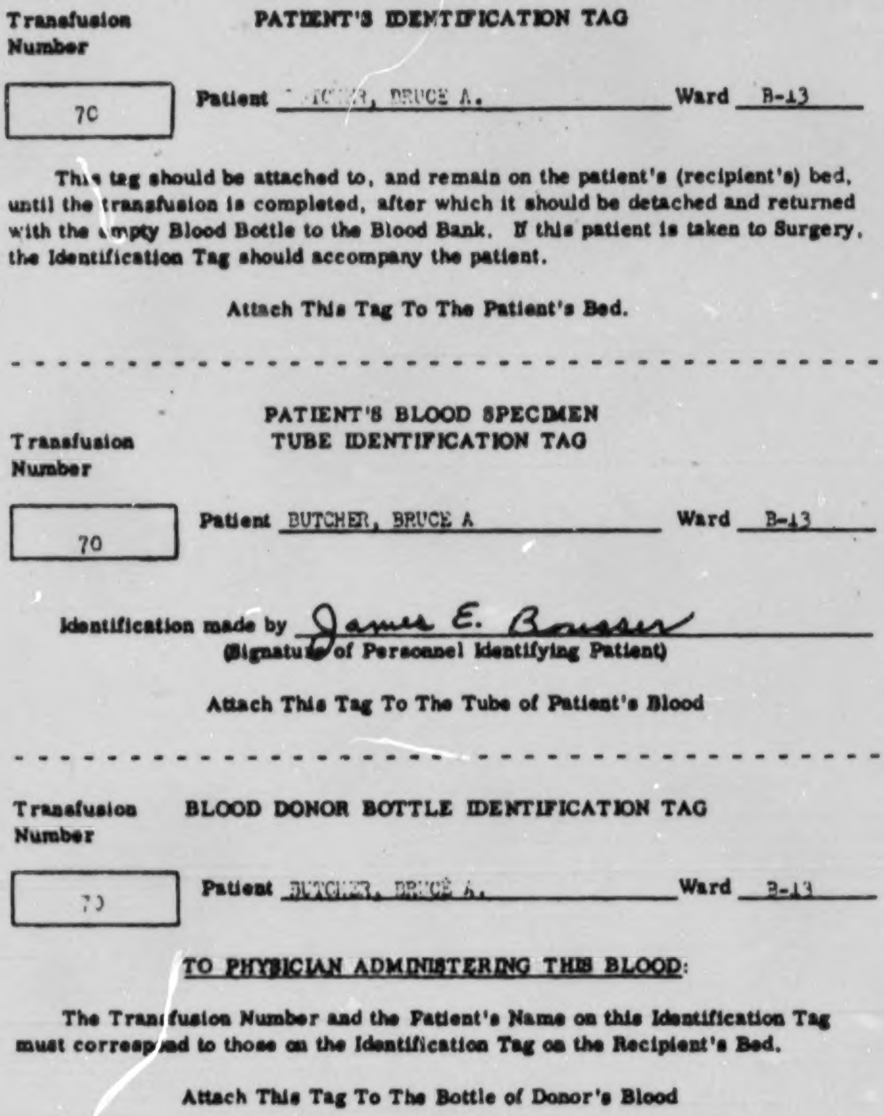
Figure 11-18. Form for investigation of transfusion reaction.

risk of a serious transfusion accident. An excellent system of identification may be established by the use of a cardboard tag divisible into three parts: (*a*) the patient's identification, (*b*) the patient's blood specimen tube identification, and (*c*) the blood donor container identification. These tags are illustrated in figure 11-19.

Additional Records

In addition to the preceding records, the following should also be considered for use by the blood bank:

1. Record of bacteriological studies.
2. Record of refrigeration temperatures.
3. Record of blood inspection.
4. Record of blood received from outside sources.
5. Laboratory work record.



Transfusion Number 70 **PATIENT'S IDENTIFICATION TAG**

Patient BUTCHER, BRUCE A. **Ward** B-13

This tag should be attached to, and remain on the patient's (recipient's) bed, until the transfusion is completed, after which it should be detached and returned with the empty Blood Bottle to the Blood Bank. If this patient is taken to Surgery, the Identification Tag should accompany the patient.

Attach This Tag To The Patient's Bed.

- -

Transfusion Number 70 **PATIENT'S BLOOD SPECIMEN TUBE IDENTIFICATION TAG**

Patient BUTCHER, BRUCE A **Ward** B-13

Identification made by James E. Bousser
(Signature of Personnel Identifying Patient)

Attach This Tag To The Tube of Patient's Blood

- -

Transfusion Number 70 **BLOOD DONOR BOTTLE IDENTIFICATION TAG**

Patient BUTCHER, BRUCE A. **Ward** B-13

TO PHYSICIAN ADMINISTERING THIS BLOOD:

The Transfusion Number and the Patient's Name on this Identification Tag must correspond to those on the Identification Tag on the Recipient's Bed.

Attach This Tag To The Bottle of Donor's Blood

Figure 11-19. Identification tags.

SECTION IV - ANNEXES

ANNEX D

BASIC PROCEDURES IN CLINICAL CHEMISTRY

OBJECTIVE: Given a series of prepared specimens of simulated body fluids having constituent concentrations that represent clinical values, test each specimen utilizing the procedures specified to attain satisfactory values, to include mathematical calculations of solution preparation and related problems.

Subject and LP No	Lesson Objective	References
Introduction to Clinical Chemistry 08-280-201	Explain clinical chemistry, objectives, and student requirements. Locate the safety equipment available in the clinical chemistry laboratory and be familiar with its proper use. Given a list of elements, valences, and abbreviations, correctly identify elements and their valences. Define element, compound, radical, ion, anion, cation, electrolyte and non-electrolyte. Identify the two types of bonding and the types of compounds formed by each. List the chief cation and anion for the two major divisions of body water compartments. Describe hypotonic, isotonic and hypertonic solutions in relation to solute concentration inside a cell and describe the effect each type of solution has on a cell.	AHS Study Guide 803, chap 1-3; Sackheim, chap 1-14.
Mathematics and Solutions 08-280-205	List the basic metric units of measurement and their respective abbreviations. With practice, interconvert units in the metric system and position decimals correctly. With practice, convert temperature values from the Centigrade to Fahrenheit and the Fahrenheit to Centigrade scales. Define solution, solute, and solvent.	Plane and Sienko, pp 17, 103-110, 117-119, 128-129, 193-195, 340. CRC Handbook, Section F, Encyclopedia Britannica, Vol 15, pp 310-314.

Subject and LP No	Lesson Objective	References
	Define the three types of percent solutions. With practice, solve percent solution problems. Define formula weight, gram molecular weight, and molar solution. With practice, calculate the formula weight and the gram molecular weight of any given compound. With practice, solve molar solution problems. Define gram equivalent weight and normality. With practice, solve gram equivalent weight and normality problems. With practice, convert normality to molarity and molarity to normality when given the formula and the problem. Define milliequivalent per liter (mEq/L) solution. With practice, solve problems involving milliequivalent per liter solutions. Define dilution and dilution factor. With practice, calculate the dilution factor of an original solution, given the dilution procedure used to achieve the final solution. With practice, calculate the volume of stock solution needed to prepare a dilute solution. With practice, perform titrations accurately, and calculate unknown concentrations based on the results of titrations. With practice, select and correctly use the appropriate formula for a given problem.	
Spectrophotometry 08-230-210	Select the six main parts of the spectrophotometer and the main function of each part. Based on Beer's Law, calculate sample concentrations and select the two	Lee, chap 4; Henry, pp 1-47; Tietz pp 103-19.

Subject and LP No	Lesson Objective	References
	factors proportional to absorbance. Select the definition of selective absorption. Given a standard curve and unknown absorbances determine unknown concentrations. Determine the absorbance of unknown samples by using a spectrophotometer, to include calibrating, zeroing, and blanking of the instrument.	
Specimen Processing and Volumetric Glassware 08-280-215	Select the most common use of volumetric flasks in clinical chemistry. Select the two types of calibration used on laboratory glassware. Select the types of pipets, the use, type of calibration, and the method of delivery for each. With practice, correctly use volumetric glassware and equipment to prepare reagents and perform laboratory determinations. Recognize the difference between serum and plasma. Select the method(s) for processing blood to obtain serum or plasma.	Tietz pp 7-16, 42-46.
Quality Control 08-280-217	Recognize the definitions of quality control, standard, control, reagent blank, accuracy, precision, and mean. Given a quality control graph, select the acceptable limits of variation from the mean of control values, and recognize which of the following abnormal conditions has occured: out of control values, shift, and/or trend. Select three possible sources of error and items to be monitored in the clinical laboratory. With practice, plot control values on a quality control	CRC Handbook of Lab Data pp 111-132, 153-173; Tietz, chap 2

550

Subject and LP No	Lesson Objective	References
	chart. With practice, use controls in laboratory procedures for monitoring the accuracy of results.	
Carbohydrates 08-280-220	Select the elements found in all carbohydrates. Select three classes of carbohydrates based on the number of simple units in each. Select the name of the reaction of glucose breakdown in the body, the end products, the forms and the primary locations of excess glucose storage. Recognize the name of the major hormone controlling blood glucose levels, and its effect on those blood glucose levels, to include the names of patient conditions. Select the preservative of choice and the alternate available method for obtaining a specimen for glucose determination. Recognize two types of specimens for glucose determination based on time of collection. Select the two body fluids tested in the Glucose Tolerance Test. Recognize three glucose test methods, and the limitations of each. Select the normal values for serum and CSF glucose concentrations. With practice, satisfactorily perform glucose determinations.	Ganong, pp 206-211, 248-266, 350; Davidsohn, pp 599-612; Tietz, pp 240-256, 1049.
Protein and Kidney Function 08-280-225	Select the four chemical elements found in all proteins. Select the function of the peptide bond in protein molecules. Recognize the four major functions of proteins in the body. Select the two major classifications of proteins and the components of each. Recognize the	Davidsohn, pp 561-569; Ganong, pp 213-216, 526-527, 211-229, 351, 513-521; Lehninger, chap 3-8.

ubject and LP No	Lesson Objective	References
	toxic and relatively non-toxic end products of protein breakdown and the respective organs responsible for the creation and removal of urea. Select the two major functions of the kidney. Select the three structural units of the nephron and the major function of each unit. Recognize the range of normal values for serum total protein and blood urea nitrogen. Recognize the clinical significance of elevated or decreased blood urea nitrogen values. With practice, satisfactorily perform blood urea nitrogen determinations.	
Chloride 08-280-230	Select the principles involved in the use of the chloridometer. Select the normal value ranges for serum and CSF chloride concentrations. Select the chief anion in the extracellular fluid. With practice, satisfactorily quantitate serum and CSF chloride concentrations.	Instr Manual Chloridomete Tietz, pp 879 884.
Practical Examination and Critique III 08-280-235	Answer all questions on the material presented in LP 08-280-205. Review examination questions and critique.	All previous references.
Practical Examination and Critique IV 08-280-240	Quantitate the serum and/or CSF concentrations of glucose, BUN and chloride with an equivalent grade of at least 70%.	All previous references.
pH and Blood Gases 08-280-250	Select the relationship between pH and hydrogen ion concentration. Select the pH ranges for acidic, basic and neutral solutions.	Harper, pp 220-228; Tietz, pp 144-145, 945-974.

ubject and P No		Lesson Objective	References
		Recognize the main function and the components of a buffer system. Recognize the description of the alkali reserve of the body. Select the enzyme involved in CO_2 transportation, the name of the phenomena in which HCO_3 and Cl^- exchange places, and the major form and location in which CO_2 is transported. Select the normal values for blood pH, HCO_3^- concentration, PO_2, PCO_2, and the ratio of concentrations of HCO_3 and H_2CO_3. Recognize the direction of the change in the HCO_3/H_2CO_3 ratio for each of acidosis and alkalosis. Recognize the two organs responsible for regulation of body pH. Select methods for quantitation of blood gases and pH. With practice, satisfactorily perform CO_2 determinations.	
Sodium and Potassium 08-260-255		Select the principle of flame photometry. Select normal ranges of serum Na and K concentrations. Select the chief cations in intracellular and extracellular fluids. With practice, satisfactorily perform sodium and potassium determinations.	Bland, pp 3-40; Tietz, pp 873-879; Davidsohn, pp 773-776; TM 8-227-6, chap 11; Lee, chap 5; Flame Photometer operator manual.
Bilirubin 08-280-260		Recognize the four basic functions of the liver. Select the substance which breaks down to bilirubin, and where in the body this breakdown occurs. Select the two basic forms of bilirubin, where they are formed, their common synonyms, and select the normal serum values of each.	Tietz, pp 1026-1038; Arnow, chap 25; Guyton, 867-868.

Subject and LP No	Lesson Objective	References
	Recognize the four types of jaundice; a related cause and type of bilirubin that is increased for each type. Select the subsequent organs involved in bilirubin excretion, and their respective roles in that excretion. With practice, satisfactorily perform serum bilirubin determinations.	
Enzymes 08-280-265	Recognize the definitions of enzyme, substrate, product, and enzyme activity. Select the six factors which influence enzyme activity. Recognize the three approaches to measuring enzyme activity. Recognize the characteristics, structural components, and function of enzymes. Select the quality of an enzyme which makes it valuable as a diagnostic tool. Select the two types of co-factors. With practice and all necessary equipment, satisfactorily perform an amylase determination.	Davidsohn, pp 822-869; Tietz, chap 12.
Laboratory Instrumentation 08-280-270	List the four advantages of using automated procedures for clinical analysis. Given a list, select basic types of separation techniques. Select the two types of multi-test analyzers. Select the six basic modules of the AutoAnalyzer and the function of each.	Technicon Manual; Tietz, pp 125-166, 188-198, 286-290, 305-315; Davidsohn, pp 502-508, 512-513, 547-560, 702-736, 782-785; Lee, pp 99-111, 118-122, 137-156, 173-197, 209-211, 243-246.

Subject and LP No	Lesson Objective	References
Written Examination and Critique V 08-280-275	Correctly answer all questions on material presented in LP 08-280-201 through LP 08-280-270. Review examination questions and critique.	All previous references.
Practical Examination and Critique V 08-280-280	Quantitate the serum concentration of sodium, potassium, carbon dioxide, and bilirubin with an equivalent grade of at least 70%.	All previous references.

SECTION IV - ANNEX D (Cont)

BIBLIOGRAPHY

Arnow, L. E. Introduction to Physiology and Pathological Chemistry. 8th ed. Saint Louis: C. V. Mosby Company, 1972.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
CHEMISTRY BRANCH

LP 08-280-201
018

General Chemistry Review

COURSES PRESENTED TO: 311-92B10

PLACE: Laboratory Sciences Division, Chemistry Branch, Academy of Health Sciences

REFERENCES:

Academy of Health Sciences US Army General Chemistry Study Guide 803 Ft Sam Houston, U.S. Army: Jun 1976. Chap 1, 2, & 3.

Sackheim, G. I. and R. M. Schultz, Chemistry for the Health Sciences 2d ed. New York: The MacMillan Co., 1975, Chap 1 - 14.

STUDY ASSIGNMENT: After Class review M 08-280-201-2

STUDENT UNIFORM AND EQUIPMENT: None

TOOLS, EQUIPMENT, AND MATERIALS: Issue one copy of M 08-280-201-2 and 26-280-209-1, page 3 before class

PERSONNEL: 1st thru 3d hour, one instructor (MOS 92B4H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, eraser, Periodic Table

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

I. INTRODUCTION (5 min)

A. Opening Statement: Welcome to Clinical Chemistry. During the next three weeks you will be learning of the role various chemicals in the body play in health and disease. In the normal person thousands of substances must be and are present in carefully controlled concentrations. Variations from the normal values can cause disease or occur in many diseases. The role of the medical laboratory technician in clinical chemistry is to quantitate these chemicals as an aid to the physician in the diagnosis of disease conditions and to help monitor the effectiveness of treatment once the diagnosis has been made.

B. Objectives

1. Explain clinical chemistry, its objectives, and student requirements.

2. Locate the safety equipment available in the chemistry laboratory and be familiar with its proper use.

C. Class Procedure and Lesson Tie-in

1. 1st hour of 120 on clinical chemistry.

2. Introduces the content and scope of the course.

II. Explanation (35 min)

A. General

1. Introduction of staff

a. OIC Chemistry

b. NCOIC Chemistry

c. BML Coordinator

d. Section leaders

e. Instructors

2. Counseling system

a. Each student assigned counselor

b. Student should consult counselor for:

(1) Academic problems

(2) Practical exercise problems

(3) Personal problems

NOTE: Insure students that personal problems will be kept in strict confidence.

c. Counselor will refer to next appropriate person if he cannot solve the problem.

B. Laboratory Safety

1. Safety hazards

   a. Caustic reagents

   b. Flammable liquids

   c. Electrical equipment

   d. Hot items

2. Safety procedures

   a. Remain calm

   b. Take necessary immediate action

   c. Report accident to instructor for follow-up treatment

3. Safety equipment

   a. Fire extinguishers

      (1) Location

      (2) Use

   b. Fire blanket

      (1) Location

      (2) Use

   c. Safety shower

      (1) Location

      (2) Use

   d. Eye wash

      (1) Location

      (2) Use

4. Emergency exit (for building or room evacuation)
    a. Alarm signal
    b. Exit route
5. Accident prevention
    a. Do not pipet by mouth
    b. Follow instructions exactly
    c. Wipe up spillage
    d. Do not tamper with electrical equipment
    e. Discard broken or chipped glassware
    f. No running in the laboratory
    g. Absolutely no horseplay

C. Grading System

1. Point breakdown
    a. Written
    b. Practical
    c. Total
2. Block I
    a. Math
    b. Glucose
    c. BUN
    d. Chloride
3. Block II
    a. Sodium (Na)
    b. Potassium (K)
    c. Bilirubin
    d. CO_2

D. Special Requirements

1. Laboratory notebook

   a. Separate from lecture notebook

   b. Used to record all test results

   c. Spiral bound stenographer's notebook ideal

NOTE: Point out the recording format mimeo and all other mimeos are contained in the folder at their desk.

2. Calculators

   a. Optional

      Ample time is allowed for manual calculations

   b. Saves time which may be used more productively

   c. Do not purchase expensive calculator

   d. Functions necessary

      (1) Add, subtract, multiply, divide

      (2) Memory helpful but not required

E. Laboratory Clean-up

   a. Limited equipment available

   b. Desk area must be clean and neat at the end of each day

   c. Report missing or damaged items to instructor

   d. Class leader will detail cleanup duties

   e. No one will be released until all duties are finished

F. Questions from Students

III. SUMMARY (5 min)

A. Review of Main Points

1. General
2. Laboratory safety
3. Grading system
4. Special requirements
5. Laboratory cleanup

B. Closing Statement: Again, welcome to chemistry. We hope that you will enjoy your time spent here. Feel free to ask questions and make suggestions about improvements in this block of instruction. The material covered in the next three weeks should provide you with a firm foundation on which to build your knowledge of clinical chemistry and will familiarize you with the basic tools available in the field.

SECOND AND THIRD HOURS

IV. INTRODUCTION (5 min)

A. Opening statement: Clinical Chemistry is a very specialized area of the field of chemistry. There are thousands of chemical reactions occurring at this very second in your body - millions and millions daily. Many of these reactions involve very intricate and complex pathways and yet they are all based on the same fundamental principles that apply in simple inorganic reactions. Today we're going to discuss some of these principles that form the basis for life.

B. Objectives.

1. Given a list of elements, valences, and abbreviations correctly identify the element and its valence.
2. Define: element, compound, radical, ion, anion, cation, electrolyte, and nonelectrolyte
3. Identify the two types of bonding and the types of compounds formed by each.

4. List the chief cation and anion of each of the two major compartments of body water.

5. Describe hypotonic, isotonic, and hypertonic solutions in relation to the solute concentration inside the cell, and describe the effect each type of solution would have on a cell.

C. Class procedure and lesson tie-in

This is the introduction to clinical chemistry. It will be followed by 117 hours of lecture material and practical exercise based on the principles outlined in this lecture.

V. EXPLANATION (80 min)

NOTE: Issue each student one copy of M 08-280-201-2. Point out that this mimeo follows the lesson closely and that by using it they can concentrate on the principles being discussed instead of extensive notetaking.

A. Elements

1. Definition - any substance that cannot be separated into different substances by ordinary chemical or physical means.
(basic particle - atom)
(net charge - zero)

2. Nomenclature

NOTE: Use the periodic chart in the student folder M 26-280-209-1, p 3, and the large wall table to help clarify the following information.

a. Each element has an abbreviation.

(1) 1 or 2 letters

(2) First letter always capitalized; second never capitalized.

(3) Examples

(a) hydrogen H

(b) Lithium Li

(c) Potassium K

(d) Silver AG

(4) These abbreviations form the basis for abbreviations of more complex substances

b. Periodic table

(1) Arrangement of the chemical elements in rows and columns in the order of their atomic numbers with elements having similar properties in the same columns.

(2) Arrangement

(a) Seven rows - (each row called a series or period)

(b) 18 columns - (each column called a group)

1 Group IA - alkali metals

2 Group VIIA - halogens

3 Group 0 or (VIIIA) - noble gases

QUESTION: What group is Sodium and Potassium in?

ANSWER: IA, alkali metals.

3. Structure of the atom

a. Nucleus

(1) Protons

(a) +1 charge

(b) Number of protons determines element (atomic number)

(2) Neutrons

(a) No charge

(b) Determines isotope (same element, only different weight)

b. Orbits

  (1) Electrons

    (a) -1 charge

    (b) Normally number of electrons equals number of protons

    (c) Arranged in specific configurations (shells, subshells, orbitals, etc.)

  (2) Outer electrons the same in any column of periodic table

    (a) Outer electrons determine reactive nature

    (b) There is a "preferred" number of electrons in the outer shell

    (c) The noble gases possess the "preferred" number

QUESTION: Why do Chlorine and Bromine have similar chemical properties?

ANSWER: They are both in group VII A and have the same number of electrons in their outer shells.

B. Compounds

1. Definition

a substance composed of two or more elements chemically bound.
(basic particle - molecule)
(net charge - zero)

2. Formation of compounds

a. Bonding results from elements attempting to reach "preferred" state electron configuration.

b. Charge developed by gain or loss of electrons (# protons ≠ # electrons), termed valence

c. Valence - measure of reactivity of element

d. Types of bonding

(1) Ionic bonding

(a) Two-step process

1 Formation of ions (charged particles)

a Positive ions (cation)

-caused by loss of electrons(s), with number of electrons lost determining valence of resulting ion.

NOTE: Show an example to help point out this change. One is given in the student mimeo.

b Negative ions (anion)

- caused by gain of electron(s), with number gained determining valence of resulting ion.

2 Opposite charged particles attract (anion attracts cation)

(b) Substances which exhibit ionic bonding are termed electrolytes

(2) Covalent bonding

(a) Some elements do not tend to lose or gain electrons to reach "preferred" state

(b) These elements tend to share electrons

(c) Formation of covalent bonds

1 Two elements need electrons to complete

2 Each element shares one or more of the other element's electrons to reach preferred state

3 Resulting bond very strong

NOTE: Again refer students to the example in their mimeo as you outline process.

(d) Substances which exhibit covalent bonding termed non-electrolytes (mainly organic compounds containing carbon)

C. Radicals

1. Definition -

   Two or more elements chemically bound which do not have a zero net charge
   (basic particle - ion)
   (net charge ≠ zero)

2. Formed as a result of element trying to reach "preferred" state, but this is only partially obtained.

3. Examples

   $SO_4^{=}$, HCO_3^- , NH_4^+

D. Chemical Formulas and Equations

1. Chemical formulas

   a. Combination of elemental symbols

   b. Subscripts to denote number of atoms or radicals

   c. Quantifier before molecule to indicate number of molecules.

2. Chemical equations

   a. Combination of chemical formulas to indicate

      a "before" and "after" situation

   b. General format

      reactant(s) ⟶ products(s)

c. Examples

$HCl + NaOH \longrightarrow H_2O + NaCl$

$Ag^+ + Cl^- \longrightarrow AgCl \downarrow$

$H_2CO_3 \longrightarrow H_2O + CO_2\uparrow$

d. Some reactions are reversible (indicated by arrows in both directions)

Example:

$Na^+ + Cl^- \overset{\longleftarrow}{\rightarrow} NaCl$

E. Law of Mass Action

1. All chemical reactions tend to reach "equilibrium"

2. At this point forward and reverse reactions equal

   $A + B \rightleftharpoons C + D$ (no net change in concentration of A, B, C, or D)

3. This equilibrium point usually tends to favor one direction (indicated by the larger arrow)

4. Ionization

   a. Ionic bonds tend to break down to varying degrees when the molecule is placed in water

   b. This separation tends to favor one side or the other

      (1) Strong electrolytes - favors split

      $NaCl \overset{\longrightarrow}{\leftarrow} Na^+ + Cl^-$

      (2) Weak electrolytes - favors together

      $HHCO_3 \overset{\longleftarrow}{\rightarrow} H^+ + HCO_3^-$

F. Electrolytes

1. Definition - substances which exhibit ionic bonding and so tend to disassociate into the component anion and cation when place in solution.

2. Examples
   a. Acids

   b. Bases

   c. Salts

3. Importance of electrolytes

   a. Muscle and nerve irritability

   b. Enzyme activators

   c. Regulation of pH and water balance

4. Distribution of electrolytes

   a. Body divided into main water compartments each containing specific electrolytes

      (1) Intracellular (inside the cells)

         (a) Chief cation - potassium (K^+)

         (b) Chief anion - phosphate ($HPO_4^=$)

      (2) Extracellular (outside the cells)

         (a) Two subdivisions -

         intravascular and interstitial

         (b) Chief cation - sodium (Na^+)

         (c) Chief anion - chloride (Cl^-)

   b. The distribution of these electrolytes controls water distribution

5. Electrolyte control of water

   a. Terminology

      (1) Solute - substance dissolved in a solution (substance present in smallest quantity)

      (2) Solvent - substance in which the solute is dissolved (usually water)

      (3) Osmosis - diffusion of H_2O across a semipermeable membrane

   b. Method of action

      (1) Isotonic solution

         (a) Equal solute concentration inside and outside the cell

         (b) Cell size remains constant

         (c) No net water movement

      (2) Hypotonic solution

         (a) Lower solute concentration outside the cell than inside

         (b) Water moves into cell in attempt to equalize solute concentrations

         (c) Cell swells (possibly to rupturing)

      (3) Hypertonic solution

         (a) Higher solute concentration outside the cell than inside

         (b) Water moves out of the cell in attempt to equalize the concentrations

         (c) Cell shrinks (crenates)

c. The lungs and kidneys regulate electrolyte concentration and hence water distribution in the body.

G. Organic Chemistry

1. Study of carbon-containing compounds

2. Structure of organic compounds very important (not just formula)

3. Classification based on common structural groups (functional groups).

a. Alcohols

(1) R - OH

NOTE: "R" is symbol for "Rest of molecule", or "attached group of atoms"

(2) $CH_3\ CH_2\ OH$

(3) Ethyl alcohol

b. Ether

(1) R - O - R

(2) $C_2\ H_5 - O - C_2\ H_5$

(3) Ethyl ether

c. Amine

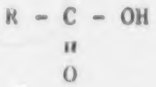
(1) $R - NH_2$

(2) $CH_3CH_2NH_2$

(3) Ethylamine

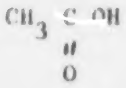
d. Carboxylic acid

(1)



$R - \underset{\underset{O}{\|}}{C} - OH$

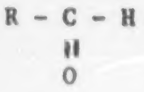
(2)



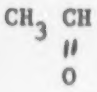
$CH_3\ \underset{\underset{O}{\|}}{C}\ OH$

(3) Acetic acid

e. Aldehyde

(1) 

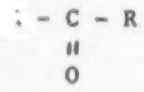
$R - \underset{\overset{\|}{O}}{C} - H$

(2) 

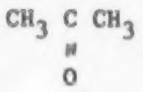
$CH_3\ \underset{\overset{\|}{O}}{C}H$

(3) Acetaldehyde

f. Ketone

(1) 

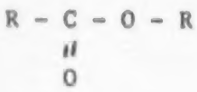
$R - \underset{\overset{\|}{O}}{C} - R$

(2) 

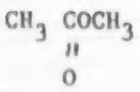
$CH_3\ \underset{\overset{\|}{O}}{C}\ CH_3$

(3) Methyl ketone

g. Ester

(1) 

$R - \underset{\overset{\|}{O}}{C} - O - R$

(2) 

$CH_3\ \underset{\overset{\|}{O}}{C}OCH_3$

(3) Methyl ester

h. Questions from Students

VI. SUMMARY (5 min)

A. Review of main points

1. Elements

2. Compounds

3. Radicals

4. Chemical equations and reactions

5. Law of Mass Action

6. Electrolytes

7. Organic Chemistry

B. Closing Statement: The basic principles outlined during the last two hours may seem too few and too basic to account for the myriad of reactions occuring in the human body, and indeed there are other principles we have not discussed, but a great many can be explained with the principles we have reviewed. During the next few weeks we will look at more of the processes with keep us healthy and our bodies functioning properly.

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LABORATORY SCIENCES DIVISION
CHEMISTRY BRANCH

General Chemistry Review

OBJECTIVES

1. Given a list of elements, valences, and abbreviations correctly identify the element and its valence.
2. Define: element, compound, radical, ion, anion, cation, electrolyte, and non-electrolyte.
3. List the two types of bonding and the types of compounds formed by each.
4. List the chief cation and anion of each of the two major compartments of body water.
5. Describe hypotonic, isotonic, and hypertonic solutions in relation to the solute concentration inside the cell and describe the effect each type of solution would have on a cell.

General Chemistry Review

A. Elements

1. Definition - any substance that cannot be separated into different substances by ordinary chemical or physical mean
   (basic particle - atom)
   (net charge- zero)
2. Nomenclature
   a. Each element has an abbreviation
      (1) 1 or 2 letters
      (2) First letter always capitalized; second never capitalized
      (3) Examples

hydrogen	H	Potassium	K
Lithium	Li	Silver	Ag

      (4) These abbreviations form the basis for abbreviation of more complex substances

b. Periodic Table

NOTE: Look at the periodic table on M 26-280-209-1 in order to more clearly see the following

(1) Arrangement of the chemical elements in rows and columns in the order of their atomic numbers with elements with similar properties in the same columns.

(2) Arrangement

(a) Seven rows - each row called a series or period

(b) 18 columns - each column called a group

1 Group I A - alkali metals

2 Group VII A - halogens

3 Group 0 or VIII A - noble gases

3. Structure of the atom

a. Nucleus

(1) Protons

(a) + 1 charge

(b) Number of protons determines element (atomic number)

(2) Neutrons

(a) No charge

(b) Determines isotope (same element only different weight)

b. Orbits

(1) Electrons

(a) - 1 charge

(b) Normally number of electrons equals number of protons

(c) Arranged in specific configurations

(2) Outer electrons determine reactive nature and properties of the element

(a) Outer electrons the same in any column of the periodic table

(b) There is a preferred number of electrons in the outer shell

(c) The noble gases have this preferred number

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B. Compounds

1. Definition - a substance composed of two or more elements chemically bound

   (basic particle - molecule)

   (net charge - zero)

2. Formation of compounds

   a. Bonding results from elements attempting to reach preferred state electron configuration.

   b. Charge develops by gain or loss of electrons (# protons ≠ # electrons) termed valence

   c. Valence - measure of reactivity of an element

   d. Types of bonding

      (1) Ionic bonding

         (a) Two step process

            1 formation of ions (charged particles)

               a Positive ions (cations)

$Na \rightarrow$ 11 protons (+11)
$\quad\ \rightarrow$ 11 electrons (-11) $\Rightarrow$ no net charge

$$Na \rightarrow Na^+ + e^-$$

$$Mg \rightarrow Mg^{++} + 2e^-$$

               b negative ions (anions)

$Cl \rightarrow$ 17 protons (+17)
$\quad\ \rightarrow$ 17 electrons (-17) $\Rightarrow$ no net charge

$$Cl \xrightarrow[e^-]{} Cl^-$$

            2 Oppposite charges attract

$$\left.\begin{matrix} Na^+ \\ Cl^- \end{matrix}\right\rangle \rightarrow NaCl$$

         (b) Substances which exhibit ionic bonding are termed electrolytes

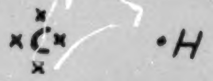
      (2) Covalent bonding

         (a) Some elements do not tend to lose or gain electrons to reach preferred state

(b) These elements tend to share electrons

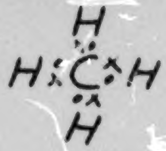
(c) Formation of covalent bonds

1 Two elements need electrons to complete preferred state configuration



2 Each element shares one or more of the other elements electrons

(d) Substances which exhibit covalent bonding termed non-electrolytes (mainly organic compounds containing carbon)



C. Radicals

1. Definition - two or more elements chemically bound which do not have a zero net charge (basic particle - ion) (net charge - zero).

2. Formed as a result of element trying to reach preferred state but this is only partially obtained.

3. Examples

$SO_4^{=}$, HCO_3^-, NH_4^+

D. Chemical formulas and equations

1. Chemical formulas

a. Combination of elemental symbols

$NaCl$

b. Subscripts to denote number of atoms or radicals

$Cu_2\ SO_4$, $Mg(HCO_3)_2$

c. Quantifier before molecule to indicate number of molecules

$Al_2(SO_4)_3 \cdot 6H_2O$

2. Chemical Equations

a. Combination of chemical formulas to indicate a "before" and "after" situation.

b. General format

$$\text{reactant(s)} \longrightarrow \text{products(s)}$$

c. Examples

$HCl + NaOH \longrightarrow H_2O + NaCl$

$Ag^+ + Cl^- \longrightarrow AgCl\downarrow$

$H_2CO_3 \longrightarrow H_2O + CO_2\uparrow$

d. Some reactions are reversible (indicated by arrows in both directions.

Example: $Na^+ + Cl^- \rightleftharpoons NaCl$

E. Law of Mass Action

1. All chemical reactions tend to reach "equilibrium"

2. At this point forward and reverse reactions equal

$A + B \rightleftharpoons C + D$ (no net change in conc. of A, B, C, or D)

3. This equilibrium point usually tends to favor one direction (indicated by the larger arrow)

4. Ionization

a. Ionic bonds tend to break down to varying degrees when the molecule is placed in water.

b. This separation tends to favor one side or the other.

(1) Strong electrolytes - favors split

$NaCl \rightleftharpoons Na^+ + Cl^-$

(2) Weak electrolytes - favors together

$HHCO_3 \rightleftharpoons H^+ + HCO_3^-$

F. Electrolytes

1. Definition - substances which exhibit ionic bonding and so tend to disassociate into the component anion and cation when placed in solution.

2. Examples

a. Acids

b. Bases

c. Salts

3. Importance of electrolytes

   a. Muscle and nerve irritability

   b. Enzyme activators

   c. Regulation of pH and water balance

4. Distribution of electrolytes

   a. Body divided into main water compartments each containing specific electrolytes

      (1) Intracellular (inside the cells)

         (a) Chief cation - potassium (K^+)

         (b) Chief anion - phosphate (HPO_4^-)

      (2) Extracellular (outside the cells)

         (a) Two subdivisions - intravascular and interstitial

         (b) Chief cation - sodium (Na^+)

         (c) Chief anion - chloride (Cl^-)

   b. The distribution of these electrolytes controls water distribution

5. Electrolytes control of water

   a. Terminology

      (1) Solute - dissolved substance in a solution (substance present in smallest quantity)

      (2) Solvent - substance in which the solute is dissolved (usually water)

      (3) Osmosis - diffusion of H_2O across a semi-permeable membrane

   b. Method of action

      (1) Isotonic solution

(a) Equal solute concentration inside and outside the cell

(b) Cell size remains constant

(c) No net water movement

(2) Hypotonic solution

(a) Lower solute concentration outside the cell than inside

(b) Water moves into cell in attempt to equalize solute concentrations

(c) Cell swells (possibly to rupturing)

(3) Hypertonic solution

(a) Higher solute concentration outside the cell than inside

(b) Water moves out of the cell in attempt to equalize the concentrations

(c) Cell shrinks (crenates)

G. Organic Chemistry - study of carbon containing compounds.

CLASS OF COMPOUNDS	ALCOHOL	ETHER	AMINE	CARBOXYLIC ACID	ALDEHYDE	KETONE	ESTER
GENERAL STRUCTURE	R–OH	R–O–R	$R{-}NH_2$	$R{-}\overset{O}{\overset{\|}{C}}{-}OH$	$R{-}\overset{O}{\overset{\|}{C}}{-}H$	$R{-}\overset{O}{\overset{\|}{C}}{-}R$	$R{-}\overset{O}{\overset{\|}{C}}{-}$
	ROH	ROR	RNH_2	RCOOH	RCOH	RCOR	RCOOR
PRODUCT OR COMPOUND	CH_3CH_2OH	$C_2H_5OC_2H_5$	$CH_3CH_2NH_2$	$CH_3\overset{O}{\overset{\|}{C}}OH$	$CH_3\overset{O}{\overset{\|}{C}}H$	$CH_3\overset{O}{\overset{\|}{C}}CH_3$	$CH_3\overset{O}{\overset{\|}{C}}OCH$
NAME IN COMMON SYSTEM	ETHYL ALCOHOL	ETHYL ETHER	ETHYLAMINE	ACETIC ACID	ACETALDEHYDE	METHYL KETONE	METHY ESTER

Periodic Table

1 H 1.00797	2 He 4.0026

LIGHT METALS — TRANSITION HEAVY METALS — NON METALS — INERT GASES

IA	IIA	IIIB	IVB	VB	VIB	VIIB	VIII			IB	IIB	IIIA	IVA	VA	VIA	VIIA	VIIIA
																	2 He 4.0026
3 Li 6.939	4 Be 9.0122											5 B 10.811	6 C 12.01115	7 N 14.0067	8 O 15.9994	9 F 18.9984	10 Ne 20.183
11 Na 22.9898	12 Mg 24.312											13 Al 26.9815	14 Si 28.086	15 P 30.9738	16 S 32.064	17 Cl 35.453	18 Ar 39.948
19 K 39.102	20 Ca 40.08	21 Sc 44.956	22 Ti 47.90	23 V 50.942	24 Cr 51.996	25 Mn 54.9380	26 Fe 55.847	27 Co 58.9332	28 Ni 58.71	29 Cu 63.54	30 Zn 65.37	31 Ga 69.72	32 Ge 72.59	33 As 74.9216	34 Se 78.96	35 Br 79.909	36 Kr 83.80
37 Rb 85.47	38 Sr 87.62	39 Y 88.905	40 Zr 91.22	41 Cb 92.906	42 Mo 95.94	43 Tc 99	44 Ru 101.07	45 Rh 102.905	46 Pd 106.4	47 Ag 107.870	48 Cd 112.40	49 In 114.82	50 Sn 118.69	51 Sb 121.75	52 Te 127.60	53 I 126.044	54 Xe 131.30
55 Cs 132.905	56 Ba 137.34	57-71 LANTHANIDE SERIES	72 Hf 178.49	73 Ta 180.948	74 W 183.85	75 Re 186.2	76 Os 190.2	77 Ir 192.2	78 Pt 195.09	79 Au 196.967	80 Hg 200.59	81 Tl 204.37	82 Pb 207.19	83 Bi 208.980	84 Po 210	85 At 210	86 Rn 222
87 Fr 223	88 Ra 226	89- ACTINIDE SERIES															

LANTHANIDE SERIES	57 La 138.91	58 Ce 140.12	59 Pr 140.07	60 Nd 144.24	61 Pm 147	62 Sm 150.35	63 Eu 151.96	64 Gd 157.25	65 Tb 158.924	66 Dy 162.50	67 Ho 164.930	68 Er 167.26	69 Tm 169.934	70 Yb 173.04	71 Lu 174.97
ACTINIDE SERIES	89 Ac 227	90 Th 232.038	91 Pa 231	92 U 238.03	93 Np 237	94 Pu 242	95 Am 243	96 Cm 247	97 Bk 247	98 Cf 249	99 Es 254	100 Fm 253	101 Mv 256	102 No 254	103 Lw 257

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-205
LABORATORY SCIENCES DIVISION 116
CHEMISTRY BRANCH

AM 5666

General Chemistry Review (9 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic) Course

PLACE: Classroom

REFERENCES:

Plane, Robert A., and Sienko, Michael J. *Chemistry*. 2nd ed. New York: McGraw Hill Book Company, Inc., 1961, pp 17, 103-110, 117-119, 128-129, 193-195, 340.
Weast, Robert C. *Handbook of Chemistry and Physics*. 48th ed. Cleveland: The Chemical Rubber Company, 1967, Sec. F.
Encyclopedia Brittanica. Chicago: William Benton, 1971, Vol. 15, pp 310-314.

STUDY ASSIGNMENT: After 1st hour, complete M 08-280-205-2. After 2nd hour, complete M 08-280-205-5, part A. After 3rd hour, review M 08-280-205-3 and complete M 08-280-205-5, part B. After 5th hour, review M 08-280-205-3 and complete M 08-280-205-5, part C. After 6th hour, review M 08-280-205-3 and complete M 08-280-205-5, part D. After 8th hour, complete M 08-280-205-5, part E.

STUDENT UNIFORM AND EQUIPMENT: Calculator optional.

TOOLS, EQUIPMENT, AND MATERIALS: M 08-280-205-1, -2, -3, -5 issued one per student at the beginning of the first hour.

PERSONNEL: One primary instructor

INSTRUCTIONAL AIDS: Chalk, chalkboard, eraser, meter stick, one liter flask with water, one gram of salt

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: L (1st thru 8th hours), EX (9th hour)

FIRST HOUR

NOTE: Issue M 08-280-205-1, -2, -3, and -5 one each per student.

I. INTRODUCTION (5 min)

A. Opening Statement: According to the anthropologists, man's written history began over 5000 years ago. For as long as man has been writing, drawing, thinking, and in general forming into societies, he has needed systems of measurement to conduct trade, plan, count wealth, and so forth. It's not surprising, then, that many different systems of measurement have developed with different civilizations, and that in scientific fields, or specifically the clinical laboratory, we should concern ourselves with choosing the system that best fits our needs.

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*This lesson plan supersedes LP 08-280-205, 074.

B. Objectives

1. List the basic metric units of measurement and their respective abbreviations.

2. With practice, interconvert units in the metric system and position decimals correctly.

C. Class Procedure and Lesson Tie-in

1. First hour of nine on general chemistry review.

2. This block of instruction forms the groundwork for all mathematical calculations to be done in the clinical chemistry laboratory.

II. EXPLANATION (35 min)

A. Measurements

NOTE: Begin development of C 08-280-205-1 through B.1.

1. Types of quantities measured

a. Length

b. Volume

c. Weight

d. Concentration

2. Systems of measurement

a. English sytem (foot-pound-gallon)

b. Prerequisites of scientific system

(1) Simple

(2) Interrelated

(3) Reproducible

c. English system does not meet prerequisites

B. Metric System

1. Definitions

a. Meter (M or m)

(1) Length

(2) 1.65 X 10^6 wavelength of orange-red light of Kr^{86}

NOTE: Instructor should display meter stick to aid students in grasping the length of a meter.

b. Liter (L or l)

(1) Volume

(2) 1/1000 or .001 cubic meters

NOTE: Instructor should display liter flask to aid students in grasping the volume of a liter.

c. Gram (g)

(1) Weight

(2) 1/1000 or .001 weight of liter of water @ 20°C

NOTE: Instructor should display gram of salt (or even have prepared one gram measurements for students to hold) to aid students in grasping the weight of one gram.

NOTE: Instructor should be aware that this definition is satisfactory for BNL, although the true value is based on a prototype gram stored in Paris, France, and differs by a factor of 1.00027

NOTE: At this point, C 08-280-205-1 should be complete.

2. Comparison with prerequisites

a. Reproducible

b. Interrelated

c. Simplicity not yet determined

3. Prefix System

a. Simplicity

- (1) Same quantifies for all types of measurement
- (2) Quantifiers are all in even powers of 10
- (3) Prefix names, abbreviations and meanings

NOTE: Have students refer to M 08-280-205-1

- (4) Placement of unit line
- (5) Awareness of omitted lines (10^{-4}, etc.)

b. Interconversions

- (1) Distinguishing prefix from unit type
- (2) Recognizing prefix correctly from abbreviation
- (3) Concept of power of 10 method
- (4) Didactic method

NOTE: Method of choice for BML. Instructor should emphasize that while this method is time-consuming, it will result in fewer careless errors by students.

  - (a) Pencil point on known value prefix
  - (b) Locate desired value prefix
  - * (c) If desired up, decimal moves left; if desired down, decimal moves right

NOTE: Student retention of up-left is enhanced by instructor physically lifting some absurd object, such as a spectrophotometer, pointing out the correlation of Up Lift-Up Left.

  - (d) Walk point up or down toward desired prefix, counting steps including skipped lines
  - (e) Decimal is moved same number as counted in direction predetermined

NOTE: Have students solve example problems from Annex B, Sec A.

C. Questions From Students

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NOTE: In assigning homework, M 08-280-205-2, emphasize that while it is permissible for students to work together, it is vital that every student be able to work every problem on the homework by himself.

III. SUMMARY (5 min)

A. Review of Main Points

1. Measurements

2. Metric System

B. Closing Statement: If one wished to bake a cake, we might be tempted to tell him that all he needs are a good recipe, a reliable oven, and the right ingredients. In so advising him, we have overlooked that to be able to use the recipe he must be able to read. Similarly, as we learn how to handle problems later on in this course we will be given reliable equipment, good ingredients, and flawless recipes--but if we cannot handle the metric system and its interconversions, we will be doomed before we start. This metric system will serve as our reading to enable us to utilize all that we will learn in the days to follow.

SECOND HOUR

IV. INTRODUCTION (5 min)

A. Opening Statement: The metric system differs from the English system in another important way--the measurement of temperatures. In this hour, we will discover how the two systems compare in that respect. We will also consider for the first time another type of measurement that we mentioned before: concentration.

B. Objectives

1. With practice, convert temperature values from the Centigrade to Fahrenheit and the Fahrenheit to Centigrade scales.

2. Define solution, solute, and solvent.

3. Define the three types of percent solutions.

4. With practice, solve percent solution problems.

C. Class Procedure and Lesson Tie-in

Second of nine hours on general chemistry review

V. EXPLANATION

A. Temperatures

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1. Fahrenheit scale
   a. Freezing point of water = 32° F
   b. Boiling point of water = 212° F
2. Centigrade scale
   a. Freezing point of water = 0° C
   b. Boiling point of water = 100° C
   c. 100 divisions = Centi grade
   d. Synonym = Celsius
3. Interconversions between Fahrenheit and Centigrade

NOTE: Point out conversion formulas on back of M 08-280-205-1

   a. Select formula which generates unit desired:
      (° C = or ° F =)
   b. Substitute given value and work arithmetic
   c. Check units of answer

NOTE: Have students solve example problems from Annex B, Sec B.

B. Solutions
   1. Need for concentration measurements
   2. Terminology
      a. Solution
         (1) Definition: A mixture of different chemicals which is continuous at the molecular level
         (2) Intuitive definition: something dissolved in something else
      b. Solvent
         (1) Definition: The component of a solution present in the greatest amount, except that water, when present, is always considered the solvent

(2) Intuitive: the "something else" in which another thing is dissolved

c. Solute

(1) Definition: Any component of a solution other than the solvent

(2) Intuitive: The "something dissolved"

(3) May be liquid or solid (or gas)

(4) May be many solutes in a solution

C. Percent Solutions

1. Definition: Parts of solute per 100 parts of total solution

2. Types

a. Weight per volume (units of g/dl, mg/dl)

b. Volume per volume (units of ml/dl)

c. Weight per weight (not used in BML)

3. Problem Solving

a. General formula:

$$\text{amt}_{\text{solute}} = \text{conc}_{\text{solution}} \times \text{vol}_{\text{solution}}$$

b. Specific formula: g = (g/dl) (dl)

NOTE: Point out this is Formula #1 on M 08-280-205-1.

c. Formula variations

(1) mg = (mg/dl)(dl)

(2) ml = (ml/dl)(dl)

d. Method

(1) Write formula

(2) Substitute given concentration and volume

(3) Convert volume to dl if needed

(4) Work arithmetic

(5) Check units of answer

NOTE: Point out that regardless which of the three variations applies, the numerical answer will be the same using any of them, and only the concentration unit needs to be checked to determine the units of the answer.

NOTE: Have students solve example problems from Annex B, Sec C.

D. Questions From Students

VI. SUMMARY (5 min)

A. Review of Main Points

1. Temperatures

2. Solutions

3. Percent Solutions

B. Closing Statement: We have finished learning the Metric System and have begun work on the different units used to express concentration. By understanding these we are better prepared to proceed to other concentration units in the future.

THIRD HOUR

VII. INTRODUCTION (5 min)

A. Opening Statement: Many times in dealing with solutions, knowing the percent concentration is not enough. For example, two 30 mg/dl salt solutions can vary widely in "saltiness" depending on which salt is used in making them up. The anti-freeze in our cars is effective only in terms of the number of molecules of solute, not the total weight of solute. The osmotic pressure that can cause a blood cell to crenate or lyse depends, not on the weight of the solute, but on the number of solute particles. Consequently, we recognize a need for some concentration unit more descriptive of the number of molecules than is a percent solution. This new unit is the subject of this lecture. on molarity.

B. Objectives

1. Define formula weight, gram molecular weight, and molar solution.

2. With practice, calculate the formula weight and the gram molecular weight of any given compound.

3. With practice, solve molar solution problems.

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C. Class Procedure and Lesson Tie-in

Third of nine hours on general chemistry review

VIII. EXPLANATION (35 min)

A. Molecular Equivalency

1. Purpose: "Equivalent number of molecules"

2. Principle: Use weights of substances in proportion to the weights of their molecules

3. Formula Weight

a. Definition: weight of a molecule expressed in a.m.u.

b. Method:

NOTE: Begin development of C 08-280-205-2 through b. (7)

(1) Write in column each element of molecule

(2) Write the molecular weight of each element next to it

(3) Write all subscripts next to the elements they follow

(4) Write all parenthetical subscripts after all elements within the parenthesis

(5) Write all factors after all the elements that follow them

(6) Multiply all rows across

(7) Add the products (express in a.m.u.'s)

NOTE: Weights of elements are listed on M 08-280-20[illegible]-3

NOTE: Have students solve example problems from Annex B, Sec D.

4. Gram molecular weight

a. Proportional weight referred to in A2

b. 1 gram = 6.02×10^{23} a.m.u. (Avogadro's Number)

c. Definition: Formula weight expressed in grams

d. Synonym: mole

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e. Abbreviation: GMW

NOTE: Have students solve example problems from Annex B, Sec E.

NOTE: Emphasize answers to GMW are always expressed in grams.

B. Molar Solutions

1. Definition: the number of GMW's of solute per liter of total solution

2. Formula for problem solving

a. $g = (M)(GMW)(L)$

NOTE: Point out this is Formula #2 on M 08-280-205-1

b. "g" is the grams of solute required to make the solution.

c. 'M' is given molarity of solution

d. 'GMW' must be calculated for given chemical

e. 'L' is given volume of total solution expressed in liters

NOTE: Point out that this means a conversion might be necessary.

3. Problem Solving

NOTE: Begin development of C 08-280-205-3 through 3d.

a. Write the formula

b. Fill in the given values, including a calculated GMW

c. Convert solution volume to liters if necessary

d. Work arithmetic and express answer in grams

NOTE: Have students solve example problems from Annex B, Sec F.

C. Questions From Students

IX. **SUMMARY**

A. Review of Main Points

1. Molecular Equivalency

2. Molar Solutions

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B. **Closing Statement:** At this point, in addition to percent solutions, we have another method of expressing concentrations. This new method has allowed us to be more specific about the number of particles present in our solution. Later we shall continue to even more refined methods of expressing concentration.

FOURTH HOUR

X. INTRODUCTION (5 min)

A. Opening Statement: We have accomplished a great deal--we can describe solutions in terms of macroscopic qualities (total amounts of solutes) or in terms of microscopic qualities (number of molecules of solute). But this is still not enough! The fact is that a very large number of the solutions we are using are not used as passive bystanders but as active participants in a reaction. If we want to describe the strength (or concentration) in terms of a reaction, while it is nice to know how many molecules are there, it just does not tell us how much reaction we will get. For this purpose, we will consider a new unit of concentration: normality.

B. Objectives

1. Define gram equivalent weight and normality.

2. With practice, solve gram equivalent weight and normality problems.

C. Class Procedure and Lesson Tie-in

Fourth of nine hours on general chemistry review

XI. EXPLANATION (35 min)

A. Review of Molarity

1. GMW

2. Molarity

3. Molar solution formula

B. Reacting Equivalency

1. Purpose: "Equivalent reacting strengths"

2. Principle: Use weights of substances in proportion to their "reacting weights"

3. Total Positive Ionic Valence (TPIV)

a. Reacting strength of a single molecule

b. Method

(1) Check valence of first element listed

NOTE: Valences are listed on M 08-280-205-3

(2) If only one value is given, multiply by the element's subscript to find TPIV

(3) If more than one value is given, calculate total valence for remainder of molecule and take opposite sign

(4) Radicals must be considered as a whole and never as individual elements

NOTE: Radicals are listed on right hand side of M 08-280-205-3

NOTE: There is only one positive radical, $NH_4 = +1$

(5) Units of report: none

NOTE: Have students solve problems from Annex B, Sec G

4. Gram equivalent weight

a. Proportional weight referred to in B.2.b.

b. Definition: GMW ÷ TPIV

c. Abbreviation: GEW

d. Synonym: Equivalent (Eq)

e. Always expressed in grams

NOTE: Have students solve example problems from Annex B, Sec H.

C. Normal Solutions

1. Definition: number of GEW's of solute per liter of total solution

2. Problem solving formula

a. g = (N)(GEW)(L)

b. "g" is grams of solute needed to make the solution

c. 'N' is given normality of the solution

d. 'GEW' must be calculated for given chemical

e. 'L' is given volume of total solution expressed in liters

NOTE: Point out that this means a conversion might be necessary.

3. Problem solving

NOTE: Begin development of C 08-280-205-4 through 3d.

a. Write the formula

b. Fill in the given values

NOTE: Remind the students that to fill in GEW they must first calculate GMW and TPIV and divide.

c. Convert solution volume to liters if necessary

d. Work arithmetic and express answer in grams

NOTE: Have students solve example problems in Annex B, Sec I.

D. Questions From Students

XII. SUMMARY (5 min)

A. Review of Main Points

1. Review of molarity

2. Reacting equivalency

3. Normal solutions

B. Closing Statement: With the addition of normality, we now have three choices of methods of expressing concentration, depending on how we intend to use the solution. This is the last major type of concentration unit we will consider in this course, though in the next hour we will consider some interesting variations of normality.

FIFTH HOUR

XIII. INTRODUCTION (5 min)

A. Opening Statement: Molarity is useful for measuring some concentrations and normality for others. Occasionally, we will have a solution which has a labeled concentration in molarity, but where (for a reaction) we need to know its normality. For this, we need to know how to convert between the two units. A second desire we might have is to make life a little easier--in the clinical laboratory we spend a great deal of time with very dilute body fluids, such as .005 N K^+ or .1N Cl^-. This

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frequent encounter with small numbers inspires us to create a new unit of concentration for very dilute solutions.

B. Objectives

1. With practice, convert normality to molarity and molarity to normality when given the formula and the problem.

2. Define milliequivalent per liter (mEq/L) solution.

3. With practice, solve problems involving milliequivalent per liter solutions.

C. Class Procedure and Lesson Tie-in

Fifth of nine hours on general chemistry review

XIV. EXPLANATION (35 min)

A. Interconversions of Molarity-Normality

1. Purpose: when different information is needed

2. Formula

a. N = M X TPIV

NOTE: Point out this is conversion Formula #3 on M 08-280-205-1.

b. Alternate form: M = N ÷ TPIV

3. Method

a. Write formula

b. Calculate TPIV and substitute in given value

c. Solve for unknown

NOTE: A student may check his work by checking whether the molarity is always smaller than or equal to the normality. If it is not, he did it backwards.

NOTE: Have students solve example problems in Annex B, Sec J.

B. Review of Normality

1. TPIV

2. GEW (synonym: Eq)

3. Normal solution

4. g = (N)(GEW)(L)

C. Milliequivalent Solutions (mEq/L)

1. Definition: 1/1000 or .001 N (mN)

2. Converted as any milli to unit line

3. Problem Solving

a. Convert mEq/L to N

b. Work as any other normality problem

NOTE: Have students solve example problems in Annex B, Sec K.

D. Questions From Students

XV. SUMMARY (5 min)

A. Review of Main Points

1. Interconversions of molarity-normality

2. Review of normality

3. Milliequivalent solutions

B. Closing Statement: We have now considered all of the concentration units (and any other type of units) that are used in BML. We can describe solutions in terms of how they were made (percent), number of solute molecules (molar), how strongly they react (normal) and even for very dilute solutions in numbers of reasonable size (mEq/L). Later we'll consider what happens to these solutions when we use them in one way or another.

SIXTH HOUR

XVI. INTRODUCTION (5 min)

A. Opening Statement: We have learned to describe solutions fully: it's now time to consider what we can do with the solutions. In general, we could use the solution in a procedure, or we could use the solution to make another solution. It is these uses that we will consider now.

B. Objectives

1. Define dilution and dilution factor.

2. With practice, calculate the dilution factor of an original solution, given the dilution procedure used to achieve the final solution.

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3. With practice, calculate the volume of stock solution needed to prepare a dilute solution.

4. With practice, perform titrations accurately, and calculate unknown concentrations based on the results of titrations.

C. Class Procedure and Lesson Tie-in

Sixth of nine hours on general chemistry review

XVII. EXPLANATION (35 min)

A. Dilutions

1. Definitions

a. Dilution: addition of solvent to a solution (which decreases solute concentration)

b. Dilution factor: The ratio of original volume to total final volume expressed as 1: ___.

2. Problem solving of dilution factors

a. Write the amount of the original volume and a colon (:)

b. Write the total amount of final volume of solution

c. Reduce to 1: _____

NOTE: Point out that a solution might have several different dilution factors depending on which original volume is considered; therefore, care must be taken in noting dilution factor requested.

NOTE: Have students solve example problems in Annex B, Sec L.

3. Problem solving of dilution problems

a. $\text{Amount}_{\text{solute}} = \text{concentration X volume}_{\text{solution}}$

b. Diluting a solution does not change amount of solute

c. Product of concentration and volume must be the same before the dilution as after

d. Formula: $C_1V_1 = C_2V_2$

e. C and V refer to concentration and volume respectively

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f. Subscripts refer to the same solution

g. Method

NOTE: Begin development of C 08-280-205-5 through g. (4)

(1) Write the formula

(2) Fill in given values (substitute an x for the unknown)

NOTE: It may aid students to determine which volume goes with which concentration if it is pointed out that the word 'of' usually acts as a bridge between the corresponding values.

(3) Check that both volumes are in the same units, as are both concentrations. If not, make conversion

NOTE: It does not matter what units are used as long as they agree. Students may find it easier at this point to write the correct unit by x to remind them later on of what unit should be in their answer.

(4) Solve for the unknown

NOTE: Have students solve example problems in Annex B, Sec M.

B. Titrations

1. Definition: determining an unknown concentration by reacting the solution exactly with a solution of known concentration

2. Problem Solving

a. Same amount of reacting strength used by both solutions

b. Amount of reacting strength = N x $\text{volume}_{\text{solution}}$

c. $N_1V_1 = N_2V_2$

d. Since N is a concentration, $C_1V_1 = C_2V_2$

e. Method: identical to dilution problems

NOTE: This would not be true if concentrations were not expressed in N (or mEq/L). However, whenever we are dealing with a reaction we should be using N for concentration anyway, so this distinction is not covered.

NOTE: Have students solve example problems in Annex B, Sec N.

XVIII. SUMMARY (5 min)

A. Review of Main Points

1. Dilutions

2. Titrations

B. Closing Statement: This completes the basic material of our general chemistry review. We now have the information necessary to handle the bulk of the calculation problems encountered in the clinical chemistry laboratory.

SEVENTH AND EIGHTH HOURS

XIX. INTRODUCTION (5 min)

A. Opening Statement: Now that we have covered all the different kinds of problems encountered in the general chemistry review, we find there is ample room for confusion in deciding which formula is used with which problem. Today, we will cover some general rules to aid in that decision, and practice working various types of problems.

B. Objective

With practice, select and use correctly the appropriate formula for a given problem.

C. Class Procedure and Lesson Tie-in

Seventh and eighth of nine hours on general chemistry review

XX. EXPLANATION (20 min)

A. Think Problems

1. Key words: 'convert,' 'what is,' 'express,' etc.

2. Types:

   a. Metric conversions (includes mEq/L to N)

   b. Temperature conversions (formulae on M 08-280-205-1)

   c. Normality-molarity conversions (Formula #3)

B. Action Problems

1. Key words: 'make,' 'prepare,' 'how much,' 'dilute,' 'titrate,' 'neutralize,' etc.

2. Method

a. Identifying solutions

  (1) Quantifier

  (2) Concentration unit

  (3) Chemical

b. Count solutions

c. If two, use $C_1V_1 = C_2V_2$ (Solution Formula #4)

d. If one, use one of first three formulae that has in it the same concentration unit used in the problem.

NOTE: Point out that if the unit were mEq/L, it must first be converted to N.

NOTE: Have students select appropriate method for example problems in Annex B, Sec O.

C. Questions From Students

XXI. PRE-EXAMINATION (45 min)

A. Directions to Students

1. All reference sheets needed are included in quiz
2. All materials except calculator and pencil(s) are to be placed in drawers
3. Turn in quiz upon completion

B. Instructor's Activities

1. Issue quiz, one per student
2. Supervise students taking quiz
3. Clarify any vague points
4. Collect answer sheets from students
5. Grade completed answer sheets
6. After all quizzes completed, answer students' questions

C. Students' Activities

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1. Place identification on answer sheet

2. Answer all quiz questions as appropriate

3. Turn in answer in answer sheet to instructor

D. Critique

XXII. SUMMARY (5 min)

A. Review of Main Points

1. Think problems

2. Action Problems

3. Pre-examination

B. Closing Statement: The examination that you had today should give you a good idea of where you are with respect to the crucial examination. If your work indicated that you might encounter difficulty passing the crucial examination, your counselor or another instructor will approach you about how you might improve your skill level.

NINTH HOUR

XXIII. CRUCIAL EXAMINATION (1 hour)

A. Directions to Students

1. All reference sheets needed are included in exam

2. All materials except calculator and pencil(s) are to be placed in drawers

3. Turn in quiz and answer sheet upon completion, making sure the test booklet number and student's name are recorded on the answer sheet

B. Instructor's Activities

1. Issue examination, one per student

2. Supervise students taking quiz

3. Clarify any vague points

4. Collect answer sheets and exam booklets from students

5. Grade completed answer sheets

6. Reteach and retest as necessary

C. Students' Activities

1. Place identification and test booklet number on answer sheet

2. Answer all examination questions as appropriate

3. Turn in answer sheet and test booklet to instructor

D. Critique

ANNEX A
INSTRUCTIONAL AIDS

CHARTS

C 08-280-205-1: Systems of Measurement

Types of Units:	LENGTH	VOLUME	WEIGHT
English:	Foot	Gallon	Pound
Metric:	Meter (M)	Liter (L)	Gram (g)
Metric Definition:	$1.65 \times 10^6 \lambda$ of Kr^{86}	.001 cubic meter	weight of .001 liter of water

C 08-280-205-2: Formula Weight ($Al_2(SO_4)_3 \cdot 6H_2O$)

Step:	#1	#2	#3	#4	#5	#6
	Al	27	2			54
	S	32		3		96
	O	16	4	3		192
	H	1	2		6	12
	O	16			6	96
Step #7						450 a.m.u.

C 08-280-205-3: Molarity Problems (solution to: "How much $Al_2(SO_4)_3 \cdot 6H_2O$ is needed to prepare 40 ml of 3 M $Al_2(SO_4)_3 \cdot 6H_2O$ solution?")

Step 1: Write the formula	g = (M) (GMW) (L)
Step 2: Fill in given values	g = (3 M) (450g) (40 ml)
Step 3: Convert to liters	g = (3 M) (450g)(.04 L)
Step 4: Work arithmetic	g = 54 g

C 08-280-205-4: Normality Problems (solution to: "How much H_2SO_4 is needed to prepare 2 dl of 6 N H_2SO_4 solution?")

Step 1: Write the formula	g = (N) (GEW) (L)
Step 2: Fill in given values:	g = (6 N) (49g) (2 dl)
Step 3: Convert to liters	g = (6 N) (49g) (.2 L)
Step 4: Work arithmetic	g = 58.8 g

C 08-280-205-5: Dilution Problems: (solution to: "How much 4 M HCl is needed to prepare 2 ml of 2 M HCl?")

Step 1: Write the formula	$C_1V_1 = C_2V_2$
Step 2: Fill in given values	(4M) (X) = (2M) (2 ml)
Step 3: Check for same units	(4M) (X ml) = (2 M) (2 ml)
Step 4: Solve for unknown	Xml = 1 ml

ANNEX B
SAMPLE PROBLEMS

Section A: Metric Conversions

1. 3.27 ml = _____ L

   a. Pencil point on "milli"
   b. Locate unit line (no prefix)
   c. Since 'up', decimal moves left
   d. 1 - centi, 2 - deci, 3 - unit line
   e. Decimal moves 3 to left:

   $.\underbrace{0}_{3}\underbrace{0}_{2}\underbrace{3}_{1}.\,2\,7$

   .00327 L, Ans.

2. 837 cg = _____ dag

   a. Pencil point on "centi"
   b. Locate "deka"
   c. Since 'up', decimal moves left
   d. 1 - deci, 2 - unit line, 3 - deka
   e. Decimal moves 3 to left:

   $.\underbrace{8}_{3}\underbrace{3}_{2}\underbrace{7}_{1}.$

   .837 dag, Ans.

3. 6142.3 μm = _____ cm

   a. Pencil point on "micro"
   b. Locate "centi"
   c. Since 'up', decimal moves left
   d. 1 - skipped line 10^{-5}, 2 - skipped line 10^{-4}, 3 - milli, 4 - centi
   e. decimal moves 4 to the left:

   $.\underbrace{6}_{4}\underbrace{1}_{3}\underbrace{4}_{2}\underbrace{2}_{1}.\,3$ .61423 cm, Ans.

4. .237 hl = _______dl

   a. Pencil point on "hecto"
   b. Locate "deci"
   c. Since 'down', decimal moves right
   d. 1 - deka, 2 - unit line, 3 - deci
   e. Decimal moves 3 to the right:

   $.\underbrace{2}_{1}\underbrace{3}_{2}\underbrace{7}_{3}.$ 237. dl, Ans

5. .00043 mg = ________ ng

a. Pencil point on "milli"
b. Locate "nano"
c. Since 'down', decimal moves right
d. 1 - skipped line 10^{-4}, 2 - skipped line 10^{-5}, 3 - micro, 4 - skipped line 10^{-7}, 5 - skipped line 10^{-8}, 6 - nano
e. Decimal moves 6 to the right:

.0 0 0 4 3 0.
1 2 3 4 5 6 430. ng, Ans.

Section B: Temperature Conversions

6. 15° C = ______ $^\circ$ F

a. $^\circ F = (^\circ C \times 1.8) + 32$
b. $^\circ F = (15 \times 1.8) + 32$
$= 27 + 32$
$= 59$
c. $^\circ$F = 59° F, Ans.

7. 50° F = ____ $^\circ$ C

a. $^\circ C = \frac{^\circ F - 32}{1.8}$

b. $^\circ C = \frac{50 - 32}{1.8}$
$= \frac{18}{1.8}$
$= 10$

c. $^\circ$C = 10° C, Ans.

8. -40° C = _____ $^\circ$F

a. $^\circ F = (^\circ C \times 1.8) + 32$

b. $^\circ F = (-40 \times 1.8) + 32$
$= -72 + 32$
$= -40$
c. $^\circ$F = -40° F, Ans.

9. 5° F = _____ $^\circ$ C

a. $^\circ C = \frac{^\circ F - 32}{1.8}$

b. $^{\circ}C = \frac{5 - 32}{1.8}$

$= \frac{-27}{1.8}$

$= -15$

c. $^{\circ}C = \underline{-15^{\circ}\ C}$, Ans.

Section C: Percent Solutions Problems

10. How much $MgSO_4$ is needed to make 4 L of 6 g/dl $MgSO_4$?

1. g = (g/dl)(dl)
2. g = (6 g/dl) (4 L)
3. g = (6 g/dl) (40 dl)
4. g = 240
5. g = 240 g, Ans. (Since g/dl unit in problem)

11. How much alcohol is needed to make 30 cl of 4 ml/dl alcohol?

1. ml = (ml/dl) (dl)
2. ml = (4 ml/dl) (30 cl)
3. ml = (4 ml/dl) (3 dl)
4. ml = 12
5. ml = 12 ml, Ans. (Since ml/dl unit in problem)

12. How much NaCl is needed to make 8 ml of a 2 mg/dl saline solution?

1. mg = (mg/dl) (dl)
2. mg = (2 mg/dl) (8 ml)
3. mg = (2 mg/dl) (.08 dl)
4. mg = .16
5. mg = .16 mg, Ans. (Since mg/dl unit in problem)

13. How much toluene is needed to make 4 dl of 8 ml/dl toluene?

1. ml = (ml/dl) (dl)
2. ml = (8 ml/dl) (4 dl)
3. No conversion needed
4. ml = 32
5. ml = 32 ml, Ans. (Since ml/dl unit in problem)

14. How much $Al_2(SO_4)_3$ is needed to make 200 ml of 10 g/dl $Al_2(SO_4)_3$?

1. g = (g/dl) (dl)
2. g = (10 g/dl) (200 ml)
3. g = (10 g/dl) (2 dl)
4. g = 20
5. g = 20 g, Ans. (Since g/dl unit in problem)

Section D: Formula Weight Problems

15. Fe_2O_3

1	2	3	4	5	6
Fe	56	2	-	-	112
O	16	3	-	-	48

7) 160 a.m.u., Ans.

16. $(NH_4)_2CO_3$

1	2	3	4	5	6
N	14	-	2	-	28
H	1	4	2	-	8
C	12	-	-	-	12
O	16	3	-	-	48

7) 96 a.m.u., Ans.

17. $Ca(C_2H_3O_2)_2 \cdot 2H_2O$

1	2	3	4	5	6
Ca	40	-	-	-	40
C	12	2	2	-	48
H	1	3	2	-	6
O	16	2	2	-	64
H	1	2	-	2	4
O	16	-	-	2	32

7) 194 a.m.u., Ans.

18. H_2SO_4

1	2	3	4	5	6
H	1	2	-	-	2
S	32	-	-	-	32
O	16	4	-	-	64

7) 98 a.m.[illegible] Ans.

19. $(NH_4)_3PO_4$

1	2	3	4	5	6
N	14	-	3	-	42
H	1	4	3	-	12
P	31	-	-	-	31
O	16	4	-	-	64

7) 149 a.m.u. Ans.

Section E: Gram Molecular Weight Problems

20. $FeSO_4$

1	2	3	4	5	6
Fe	56	-	-	-	56
S	32	-	-	-	32
O	16	4	-	-	64

7) 152 a.m.u.
8) 152 g., Ans.

21. $C_6H_{12}O_6$

1	2	3	4	5	6
C	12	6	-	-	72
H	1	12	-	-	12
O	16	6	-	-	96

7) 180 a.m.u.
8) 180 g., Ans.

Section F: Molarity Problems

22. How much Na_2SO_4 is needed to make 3 dl of 2 M Na_2SO_4?

a. g = (M) (GMW) (L)
b. g = (2M)(142 g) (3 dl)
c. g = (2M)(142 g) (.3L)
d. g = 85.2g, Ans.

Na	23	2	46
S	32		32
O	16	4	64
			142g = GMW

3. How much $FeCl_3$ is needed to prepare 40 ml of 2.5 M $FeCl_3$?

a. g = (M) (GMW) (L)
b. g = (2.5M) (162.5g) (40ml)
c. g = (2.5M) (162.5g) (.04L)
d. g = 16.25g, Ans.

Fe	56		56
Cl	35.5	3	106.5
			162.5g = GMW

24. How much K_2WO_4 is needed to prepare 10 ml of 4 M K_2WO_4?

a. g = (M)(GMW)(L)
b. g = (4M)(326 g)(10 ml)
c. g = (4M)(326 g)(.01 L)
d. g = 13.04g, Ans.

K	39	2	78
W	184		184
O	16	4	64
			326g = GMW

25. How much $Ca(NO_3)_2$ is needed to prepare 3 L of 1.5 M $Ca(NO_3)_2$?

a. g = (M)(GMW)(L)
b. g = (1.5M)(164g)(3L)
c. No conversion needed
d. g = 738 g, Ans.

Ca	40			40
N	14		2	28
O	16	3	2	96
				164g = GMW

26. How much $CuNO_2$ is needed to make 1 dl of .1 M $CuNO_2$ solution?

a. g = (M) (GMW) (L)
b. g = (.1M) (110 g) (1 dl)
c. g = (.1M) (110g) (.1L)
d. g = 1.1 g, Ans.

Cu	64		64
N	14		14
O	16	2	32
			110g = GMW

Section G: Total Positive Ionic Valence (TPIV) Problems

		First Element	x Number	or Remainder of Molecule
27.	CaO	+2	1 = 2, Ans.	
28.	$Al(OH)_3$	+3	1 = 3, Ans.	
29.	NiO	+2,+3		O = -2, so 2, Ans.
30.	$Hg(OH)_2$	+1,+2		(OH = -1 x 2 = 2, Ans.
31.	K_3PO_4	+1	3 = 3, Ans.	
32.	$Ca_3(PO_4)_2$	+2	3 = 6, Ans.	
33.	$FeSO_4$	+2,+3		SO_4 = -2 so 2, Ans.
34.	$Cu_3(PO_4)_2$	+1,+2		PO_4 = -3 x 2 = 6, Ans.
35.	$(NH_4)_2SO_4$	+1 (NH_4 rad.)	2 = 2, Ans.	

Section H: Gram Equivalent Weight (GEW) Problems

36. CaO

$\text{GEW} = \dfrac{\text{GMW}}{\text{TPIV}}$, GMW:

Ca	40	
O	16	
	56 g	TPIV = 2

$\text{GEW} = \dfrac{56\text{g}}{2} = \underline{28\text{ g}}$, Ans.

37. $Al(OH)_3$

$\text{GEW} = \dfrac{\text{GMW}}{\text{TPIV}}$, GMW:

Al	27		27	
O	16	3	48	TPIV = 3
H	1	3	3	
			78g	

$\text{GEW} = \dfrac{78\text{ g}}{3}$

$\text{GEW} = \underline{26\text{ g}}$, Ans.

38. NH_4Cl

$\text{GEW} = \dfrac{\text{GMW}}{\text{TPIV}}$, GMW:

N	14		14	
H	1	4	4	TPIV = 1 (NH_4 rad.)
Cl	35.5		35.5	
			53.5 g	

$\text{GEW} = \dfrac{53.5\text{ g}}{1}$

$\text{GEW} = \underline{53.5\text{ g}}$, Ans.

Section I: Normality Problems

39. How much $FeSO_4$ is needed to prepare 4 dl of 2.5 N $FeSO_4$?

a. g = (N) (GEW) (L)
b. g = (2.5N) (76g) (4dl)
c. g = (2.5N) (76g)(.4L)
d. g = 76 g, Ans.

Fe	56		56	
S	32		32	TPIV = 2
O	16	4	64	GEW = GMW/TPIV
			152g	= 152g/2
				= 76 g

40. How much NiO is needed to make 30 ml of 4 N NiO solution?

a. g = (N)(GEW)(L)
b. g = (4N)(37.5g)(30ml)
c. g = (4N)(37.5g)(.03L)
d. g = 4.5 g, Ans.

Ni	59	59	
O	16	16	TPIV = 2
		75g	GEW = GMW/TPIV
			GEW = 75g/2
			= 37.5 g

1. How much NH_4Cl is needed to make 3 L of 8 $\underline{N}$ NH_4Cl solution?

a. g = (N)(GEW)(L)
b. g = (8N)(53.5g)(3L)
c. No conversion needed
d. g = 1284 g, Ans.

N	14		14
H	1	4	4
Cl	35.5		35.5
			53.5 g

TPIV = 1
GEW = GMW/TPIV
= 53.5g/1
53.5 g

Section J: Molarity-Normality Interconversions

42. 3 $\underline{M}$ H_2SO_4 = ______ $\underline{N}$ H_2SO_4

a. N = M x TPIV

b. N = 3 x 2

c. N = 6 N, Ans.

43. 6 $\underline{N}$ $(NH_4)_2CO_3$ = ______ $\underline{M}$ $(NH_4)_2CO_3$

a. M = N/TPIV

b. M = 6/2

c. M = 3 M, Ans.

44. 1.5 $\underline{M}$ K_3PO_4 = ______ $\underline{N}$ K_3PO_4

a. N = M x TPIV

b. N = 1.5 x 3

c. N = 4.5 N, Ans.

45. 7 $\underline{N}$ HCl = ____ $\underline{M}$ HCl

a. M = N/TPIV

b. M = 7/1

c. M = 7 M, Ans.

Section K: Milliequivalent per Liter Problems

46. How much Na_2SO_4 is needed to make 2 dl of 20 mEq/L Na_2SO_4?

a. 20 mEq/L = .02 N

b. g = (N) (GEW)(L)
g = (.02N) (71g) (2 dl)
g = (.02N)(71g)(.2L)
g = .284 g, Ans.

Na	23	2	46
S	32		32
O	16	4	64
			142 g

TPIV = 2
GEW = GMW/TPIV
GEW = 142g/2
= 71 g

47. 18 mEq/L = ______ N

Decimal 3 to left: .018 N, Ans.

48. 200 mEq/L = _____ N

Decimal 3 to left: .2 N, Ans.

49. How much Fe_2O_3 is needed to make 5 L of 2 mEq/L Fe_2O_3?

a. 2 mEq/L = .002 N

b. g = (N)(GEW)(L)
g = (.002N)(26.7g)(5L)
No conversion needed
g = .267g, Ans.

Fe	56	2	112
O	16	3	48
			160

TPIV = 6
GEW = GMW/TPIV
GEW = 160g/6
= 26.7g

50. How much H_2SO_4 is needed to make 4 ml of 3 mEq/L H_2SO_4?

a. 3 m Eq/L = .003 N

b. g = (N)(GEW)(L)
g = (.003N)(49g)(4ml)
g = (.003N)(49g)(.004L)
g = .000588 g, Ans.

H	1	2	2
S	32		32
O	16	4	64
			98g

TPIV = 2
GEW = GMW/TPIV
GEW = 98g/2
= 49 g

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Section L: Dilution Factor Problems

51. What is the dilution factor of the serum in the following dilution:

3 ml H_2O

2 ml Serum

6 ml H_2SO_4

a. 2: _______ (Amount of serum)

b. 2: 11 (Total volume)

c. 1: 5.5 Ans. (11/2 = 5.5)

52. A dilution is made by adding the following together:

40 ml H_2O

20 ml saline

30 ml specimen

What is the dilution factor of the specimen?

a. 30: ______ (Amount of specimen)

b. 30: 90 (total volume)

c. 1: 3, Ans. (90/30 = 3)

53. A serum sample is diluted as follows:

2 ml serum

3 ml water

0.5 ml HCl

2.5 ml saline

What is the dilution factor of the sample?

a. 2: ______ (Amount of sample)

b. 2: 8 (total volume)

c. 1: 4 (8/2 = 4)

Section M: Dilution Problems

54. How much 6 M HCl is needed to prepare 40 ml of 2 M HCl?

1. $C_1V_1 = C_2V_2$
2. (6M) (X) = (2M)(40 ml)
3. No conversion needed if X is in ml
4. 6X(ml) = 80
   X(ml) = $13.\overline{3}$
   X = 13.3 ml, Ans.

55. It took 2 dl of 40 mEq/L HCl to make a 10 mEq/L HCl solution. What volume of 10 mEq/L HCl was made?

1. $C_1V_1 = C_2V_2$
2. (40 mEq/L)(2 dl) = (10 mEq/L)(X)
3. No conversion needed if X is in dl
4. 80 = 10X(dl)
   8 = X (dl)
   X = 8 dl, Ans.

56. 3 L of 20 g/dl NaCl were made from 200 ml of a stronger NaCl solution. What was the concentration of the stronger solution?

1. $C_1V_1 = C_2V_2$
2. (20 g/dl)(3L) = (X)(200 ml)
3. (20 g/dl)(3000ml) = (X)(200ml) or (20g/dl)(3L) = (X)(.2L)
4. 60000 = 200X(g/dl) or 60 = .2X (g/dl)
5. 300 = X(g/dl)
   X = 300 g/dl, Ans.

57. How much 6N NaOH is needed to make 5 dl of 1 N NaOH?

1. $C_1V_1 = C_2V_2$
2. (6N)(X) = (1N)(5dl)
3. No conversion needed if X is in dl
4. 6X(dl) = 5
   X(dl) = $.8\overline{3}$
   X = .83 dl, Ans.

Section N: Titration Problems

58. How much 6 N HCl is needed to titrate 12 ml of 4 N NaOH?

1. $C_1V_1 = C_2V_2$
2. (6N) (X) = (4N)(12 ml)
3. No conversion needed if X is in ml
4. 6X(ml) = 48
   X(ml) = 8
   X = 8 ml, Ans.

[illegible]. It took 40 ml of 3 N NH_4OH to titrate 2 dl of an HCl solution. What is the concentration of the HCl?

1. $C_1V_1 = C_2V_2$
2. (3N)(40ml) = (X)(2dl)
3. (3N) (.4dl) = (XN)(2dl) or (3N)(40 ml) = (XN)(200ml)
4. 1.20 = 2X(N) or 120 = 200X(N)

.6 = X(N)
X = .6 N, Ans.

60. If 2 L of 8 mEq/L $Ca(OH)_2$ is reacted exactly with 2 N H_2SO_4, how much 2 N H_2SO_4 is required?

1. $C_1V_1 = C_2V_2$
2. (8mEq/L)(2L) = (2N)(X)
3. (8mEq/L)(2L) = (2000mEq/L)(XL) or (.008N)(2L) = (2N)(XL)
4. 16 = 2000X(L) or .016 = 2X(L)

.008 = X(L)
X = .008 L, Ans.

Section O: Formula Selection Problems

61. What is the molarity of 6 N HCl?

Type: Think ("What is")

Formula: M = N/TPIV

62. How much NaCl is needed to prepare 2 L of 6 g/dl NaCl solution?

Type: Action ("prepare")

Number of Solutions: 1 (6 g/dl NaCl)

Concentration Unit: g/dl

Formula: g = (g/dl)(dl)

63. 37° C = _____° F

Type: Think (" = ")

Formula: °F = (°C x 1.8) + 32

64. How much $MgSO_4$ is needed to prepare 4 L of 6 N $MgSO_4$?

Type: Action ("prepare")

Number of Solutions: 1 (6 N $MgSO_4$)

Concentration Unit: N

Formula: g = (N)(GEW)(L)

65. **Express 307 mEq/L in Normality.**

Type: Think ("express")

Formula: Metric Conversion (milli to unit line)

66. How much 4 N NaOH is needed to make 3 L of 2 N NaOH?

Type: Action ("make")

Number of Solutions: 2 (4 N NaOH, 2 N NaOH)

Formula: $C_1V_1 = C_2V_2$

67. What is the dilution factor of the serum in:

3 ml serum

2 ml H_2O

Type: Think ("What is")

Formula: None (Dilution Factor -- 2: ___, 2:5, 1:2.5)

68. How many grams of Fe_2O_3 are needed to make 3 L of 8 M Fe_2O_3?

Type: Action ("make")

Number of Solutions: 1 (8 M Fe_2O_3)

Concentration Unit: M

Formula: g = (M) (GMW) (L)

69. It took 30 ml of 6 N NaOH to titrate 4 dl of an unknown HCl solution. What is the concentration of the HCl?

Type: Action ("titrate")

Number of Solutions: 2 (6 N NaOH, unknown HCl solution)

Formula: $C_1V_1 = C_2V_2$

70. How much H3PO$_4$ is needed to prepare 4 ml of a 10 mEq/L H_3PO_4?

Type: Action ("prepare")

Number of Solutions: 1 (10 mEq/L H_3PO_4)

Concentration Unit: mEq/L

Formula: g = (N) (GEW) (L) (mEq/L must first be converted to N)

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CHEMISTRY BRANCH

General Chemistry Review

According to the anthropologists, man's written history began over 5000 years ago. For as long as man has been writing, drawing, thinking, and in general forming into societies, he has needed systems of measurement to conduct trade, plan, count welath, and so forth. It is not surprising, then, that many different systems of measurement have developed with different civilizations, and that in scientific fields, or specifically the clinical laboratory, we should concern ourselves with choosing the system that best fits our needs.

What sort of things do we commonly measure? Length, weight, and volume, certainly. Also, especially when we are in the laboratory and frequently dealing with liquids, we want to measure the quantity of a substance in a solution, or its concentration. The ways in which we measure concentrations will be the subject of future lectures. Now, we will consider only the first three things: length, weight, and volume.

In measuring these things, we could use a variety of systems. The one we are most familiar with is the English or American system, in which we measure length in feet, volume in gallons, and weight in pounds. In deciding whether this, or any, system is the best for us, we want to ask three questions: Is the system simple? Is it easy to go from big units to little units, or vice versa? Second, Is the system interrelated? If we have one type of measurement (such as length), can we figure a second or a third type (such as volume or weight)? Third, is the system reproducible? Can a scientist in a far off place come up with the exact same size for a given unit as we have?

Does the English system satisfy these questions? Is it simple? How many pints are in a quart? Quarts in a gallon? Tablespoons in a cup? Feet in a mile? Ounces in a pound? Obviously there is no simple rule for going from big units to little units or vice-versa. Is the English system interrelated? How many cubic feet in a gallon? If one has a measure for a gallon, how could he calculate or discover the weight of a pound? No interrelation. Is the system reproducible? If you had no ruler, how could you find the exact length of a foot? The size of a gallon? The weight of a pound? Apparently, the English, or foot-gallon-pound, does not satisfy any of our three questions.

The system of choice in the laboratory is called the metric system. As we examine the units in it, we will discover it totally satisfies our three prerequisites. The metric system takes its name from its basic unit of length, called the meter and abbreviated 'm.' This length is exactly reproducible because of the way in which it is defined. Instead of referring to a physical object, we refer the length of the meter to light. Light travels in waves not totally unlike ocean waves, but unlike ocean waves, the distance between waves for any given color is exactly the same. Therefore, to get a specific, never changing length, we need only select a specific color. The color we select is the orange-red color given off by Krypton 86. However, this is a very tiny length--too small to use for most things. Therefore, we take a large number of these

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*This instructor's manuscript supersedes IM 08-280-205, 074.

lengths, 1,650,000, and that is how we define the meter. To define our basic unit of volume, we make a cube one meter long on each edge. Since this is a fairly large volume, we take one one-thousandth of it and define that as the liter, abbreviated 'l' or 'L.' Finally, we fill our liter with distilled water to get a definite weight, and again take one one-thousandth of this weight for our basic unit the gram, abbreviated 'g.' This completes three of the four types of basic units we mentioned. We can notice from a chart of their relationships that they are all interrelated, and since the meter is reproducible, so are they all. Now we must consider whether this system is simple.

In the metric system, all varying sizes of the same type of unit include the basic unit in their names. For example, any unit of weight, no matter how big or how small, will still have gram as the last part of its name. These units differ only by their prefixes, which tell how they relate to the basic unit (such as the prefix 'deci' in decigram, which means one tenth of a gram). To make life even simpler, all prefixes are the same in meaning regardless of the type of unit being measured (a deciliter is one tenth of a liter; a decimeter is one tenth of a meter). Another nice thing is that all the units refer to exact multiples of ten--one hundred, one thousand, one millionth, etc. The prefixes we will use in this course are nano (n), micro (μ), milli (m), centi (c), deci (d), deka (da), hecto (h), kilo (k), and representing 10 raised to the powers of -9, -6, -3, -2, -1, 1, 2, and 3 respectively. Note that there is no 0 power, since this is the basic unit itself (meter, gram, liter) and that certain powers of 10 do exist but are not included (-8, -7, -5, -4).

The primary type of problem you will encounter in this course is one in which you are given a measurement in one size unit and asked to give the same measurement in another size unit, such as 30 ml = ____hl? To work such problems, you must recognize that the last letter (l) always refers to the basic type of unit (liter), and the first letter(s) to the prefix. You must also be familiar with the different abbreviations to be able to distinguish between similar looking prefixes (d and da). Those gifted in math can probably work such conversions easily by multiplying by 10 to the power of the difference in the exponents, but for the rest of us we recommend the following method. This method is somewhat time-consuming, but aids greatly in reducing careless errors that make for wrong answers. First, place your pencil point on the chart on the prefix whose value is given (m). Second, scan the page for the desired prefix and note whether it is up or down from your pencil. If it's up, we will be moving the decimal left, if it is down, we will be moving the decimal right. You may find this easier to remember if you picture lifting up a spectrophotometer; lift and up go together, and so do up and left. In this case it is up, so we will be moving the decimal left. Next, move your pencil up (or down) the page, stopping at each line, and counting as you do so, until the desired prefix is reached. Don't forget to count the skipped lines if you hit them, including the unit line. (In this case, we count five steps). Finally move the decimal the same number of steps you counted (5) in the direction already decided upon (left) and you get the desired answer (.0003 hl).

If one wished to bake a cake, we might be tempted to tell him that all he needs are a good recipe, a reliable oven, and the right utensils and ingredients.

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n so advising him, we have overlooked that he must be able to read! Similarly, as we learn how to handle problems later on in this course, we will be given reliable equipment, good ingredients, and flawless recipes--but if we cannot handle the metric system and its interconversions, we will be doomed before we start. The ability to do interconversions in the metric system will serve as our ability to read, enabling us to utilize all that we will learn in the days to follow.

The metric system differs from the English system in another important way--the measurement of temperatures. We will discover how the English and metric systems compare in that respect. We will also consider for the first time another type of measurement that we mentioned earlier: concentration.

We have long been familiar with degrees Fahrenheit, in which water freezes at 32° and boil at 212°. If these numbers seem a bit unusual or arbitrary, its only because they are. In the Centigrade or metric system of measurement, water freezes at 0° C. and boils at 100° C. Since that makes 100 gradations between freezing and boiling the scale is called centi grade. Sometimes this scale is also referred to as Celsius, after its originator, but in either case is abbreviated C. To convert from one temperature scale to the other is a simple case of using the formulas on the back of M 08-280-205-1. To decide which of the two formulae should be used, notice what is the scale being asked for (such as 37° F = _____ $^\circ$C. asks for C). Use the formula that has that same scale on the left side ($^\circ C = (^\circ F - 32) \div 1.8$). Then simply plug in the given temperature and solve: ($^\circ C = (37-32) \div 1.8 = 5 \div 1.8 = 2.8^\circ$ C). The answer you give must include both the degree sign ($^\circ$) and the scale abbreviation (C or F).

What are some types of specimens we might receive in the clinical laboratory? Serum, blood, sputum, cerebrospinal fluid, urine, feces, etc. The vast majority of these are liquids, and in most cases it is not important to us what the total volume of the liquid specimen is. Instead, we are concerned with what is in the solution--what quantities of things are there in a given amount of solution. For instance, in hematology we learned of a test to discover how much hemoglobin is present in blood. This need to express quantities of things in a solution results in our need for a fourth type of metric unit: concentration.

A solution is defined as a mixture of different chemicals which is continuous at the molecular level. We normally think of a solution as dissolving something in something else. With this in mind, we would say a solution is composed of two major parts: the something dissolved, and the something it's dissolved in. A solvent is the component of a solution present in the greatest amount. It is the something we dissolved a substance in. The exception to the definition is water--it is considered the universal solvent, and is the solvent anytime it is present, whether it has the greatest amount or not. We are already somewhat familiar with this practice as in the case of a very high proof whiskey, which we might describe as 160 proof. Even though there is four times as much alcohol as water in it, we are still describing it in terms of water as the solvent. A solute is any component of a solution which is not the solvent. This is the "something dissolved." Notice, then, that the solute could be a solid or a liquid, or even a gas. There might be just one solute in a solution or several.

We are now ready to consider a type of concentration unit which we categorize

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as percent solutions, defined as parts of solute per 100 parts of total solution. This definition is a bit ambiguous since a solute could be a solid or liquid (we will not consider gaseous solutes in this course), and the total solution could be measured in weight or volume, so we have more than one form for a percent solution concentration unit, and three general types of percent solutions. These three types are weight per volume, volume per volume, and weight per weight. We will not use weight per weight solutions in this course. Most of the solutions and specimens we have in the laboratory we measure in milliliters. This will hold true for the volume of our percent solutions. But according to our definition, we are talking about 100 parts of total solution, or 100 ml. What unit = 100 ml? Deciliter, or dl. So, regardless of whether we are talking about weight per volume or volume per volume percent solutions, our denominator of the unit will be dl. For a volume to volume, we will follow our practice of measuring liquids in ml, so our unit is ml/dl (ml of solute per dl of total solution). For weight to volume, we will measure in grams (g) for moderate amounts and milligrams (mg) for small amounts, recalling that 1 mg = 1/1000 g. So, in this course all percent solutions will be expressed as either ml/dl, g/dl, or mg/dl.

In general the only types of problems you will be responsible for in per cent solutions are ones in which you are asked to determine how much solute is needed to make up a given volume of a certain concentration solution, such as "How much NaCl is needed to prepare 4 liters of 12 g/dl NaCl solution?" The general method we would use to work a problem like this is:

Amount of solute = concentration X volume of solution desired

Remembering that in this case our concentration unit is g/dl, and that we always measure total solution in dl, this formula could be condensed to read:

$$g = (g/dl)(dl)$$

Notice that if our concentration unit gives "parts of solute" in grams, then our answer must be in grams. Similarly, for other concentration units the formula would change to:

$$mg = (mg/dl)(dl)$$
$$ml = (ml/dl)(dl)$$

To work the problem, we simply write the formula, plug in 12 g/dl for the concentration and 4 L for dl--and stop right there. Notice that the formula calls for dl for total solution--not L. Therefore, we need to convert 4 l to 40 dl. Now we can proceed with the multiplication to get our answer of 480 g. If we has accidentally used one of the other two formulas for this problem, our answer would still have been 480, but not in grams. So the only difference the particular concentration unit makes in working these problems is in determining what answer units we will have. However, the units given for desired volume are very important. They must be first converted to deciliters to get the right answer.

We have finished learning the metric system and have begun work on the different units used to express concentration. By understanding these, we are better prepared to proceed to other concentration units in the lectures to follow.

Many times in dealing with solutions, knowing the percent concentration is not enough. For example, two 30 mg/dl salt solutions can vary widely in "saltiness," depending on which salt is used in making them up. The anti-freeze in our cars is effective only in terms of the number of molecules of solute, not the total weight of the solute. The osmotic pressure that can cause a blood cell to crenate or lyse depends not on the weight of the solute but on the number of solute particles. Consequently, we recognize a need for some concentration unit more descriptive of the number of molecules than is percent solutions. This new unit is the subject of this lecture on molarity.

The first step in obtaining our new concentration unit is to consider what we want to accomplish. We want to able to say something about the number of molecules in a solution. Before we attempt that, let us try to say something about the number of molecules in a solid; and to find some way of treating solids where we can tell how much of each different chemical would be needed to get consistently the same number of molecules. This would be a "molecular equivalency."

If I had 100 pounds of bricks, each weighing ten pounds, it would be easy to figure that I had ten bricks. However, if I also had some smaller bricks, each weighing five pounds, how much weight of the bricks would I need to know that I had the same number of bricks? Fifty pounds. So in general, if I wanted to keep my same number of bricks (10), regardless of their weight, I need only multiply by my desired number the weight of each type of brick, or to take proportional weights always ten times each brick's weight. This same principle applies to chemicals. Each chemical is made up of identical molecules (bricks) which vary in weight from chemical to chemical. These molecular weights are extremely small--so small that when we try to measure them we forget about adding a prefix to gram and use a very tiny unit called the atomic mass unit or a.m.u. All molecular weights are expressed in a.m.u.'s. Each molecule is in turn made up of atoms, so that if we know the weight of each atom, we could simply add up all the atoms in a molecule to find the weight of a molecule. For example, if we wished to figure the weight of $Al_2(SO_4)_3 \cdot 6H_2O$, these are the steps we would follow. First we would write in a column each element listed in order. Then using a table of atomic weights, we write the weight of each atom next to it. Next we write all subscripts (the numbers slightly below the line) on the same rows as the elements which they follow; however, if a subscript is after a parenthesis like the 3 above, we write it after all the elements within the parenthesis (S, and the first O). Next for all factors (numbers on the same line) we write them on the same line as all the elements that follow them (6 above on line with H and the second O). Finally we multiply each row across and add the products to get the molecular weight. This is called the Formula Weight and is always expressed in a.m.u.'s. These are our bricks and we can now determine for each chemical what its basic building block weighs.

Our next step is to choose a consistent number of molecules to compare chemicals with (such as we always had 10 bricks in each stack, regardless of its weight). But here we will make life easy--our basic number of molecules will be 6.02×10^{23}! This is called Avogadro's Number. But why is it easy? It is easy because we do not need to do any multiplying at all! It so happens that one gram equals 6.02×10^{23} a.m.u., so to get our consistent number of molecules, we need only take the formula weight and express it in grams. This is called the Gram Molecular Weight, and is defined as the formula weight expressed in grams (not a.m.u.). It is abbreviated GMW and occasionally called by a shorter name, mole. Hence, one gram molecular weight of $Al_2(SO_4)_3 \cdot 6H_2O$ is not 450 a.m.u. but 450 grams. Remember than that the Gram in Gram Molecular Weight is to remind you that the answer must be expressed in grams.

At this point, we realize that if we take the GMW of any chemical we always have the same exact number of molecules. If we then dissolve this chemical in a liter of water, we will still have the same exact number of molecules of solute, and in general, we could describe how many molecules are in a solution by stating how many GMW's were dissolved in each liter of solution. This is our new unit of concentration, molarity, defined as the number of GMW's per liter of total solution, and abbreviated M.

Our principle concern for molar solutions in this course is the same as it was for percent solutions. How much solute is needed to prepare a given volume of solution of a given molarity? For example: "How much $Al_2(SO_4)_3 \cdot 6H_2O$ is needed to prepare 40 ml of 3 M. $Al_2(SO_4)_3 \cdot 6H_2O$ solution? Our formula to answer that question is:

$$g = (M)(GMW)(L)$$

where M is the given molarity (3); GMW is the calculated gram molecular weight (450 grams); and L is the desired volume of the solution (40 ml). Our first step is to write the formula. Next we plug in the given and calculated values, and then we stop. The equation requires the desired volume to be in liters--if it is not, then we must convert it before we go on (40ml = .04 L). After the conversion we need only do the multiplication and express our answer in grams (54 grams).

At this point, in addition to percent solutions, we have another method of expressing concentration. This new method has allowed us to be more specific about the number of particles present in our solution. Next, we shall continue to even more refined methods of expressing concentrations.

In the past we have accomplished a great deal--we can describe solutions in terms of macroscopic qualities (total amounts of solutes) or in terms of microscopic qualities (number of molecules of solute). But this is still not enough! The fact is that a very large number of the solutions we are using are not used as passive bystanders but as active participants in a reaction. If we want to describe the strength (or concentration) in terms of a reaction, while it is nice to know how many molecules are there, it just does not tell us how much reaction we will get. For this purpose, now we will consider a new unit of concentration: Normali

Since there is a major relationship between normality and molarity, let us take a second to review the last lecture. A GMW stands for a Gram Molecular Weight and is the Formula Weight of a chemical expressed in grams. A Formula Weight is the sum of the weights of the atoms in a molecule expressed in a.m.u. A molar solution is given in the number of gram molecular weights per liter of total solution.

Our goal with normality is not molecular equivalency, but reacting equivalency. In other words, we wish to be able to say that given two solutions of identical normality, they will react to exactly the same extent. How do we go about it?

How many pies can six identical men eat in an hour? To answer this we would need to know how many pies one of the men could eat in an hour. This same principle applies to chemicals. To figure out how much reacting a certain number of molecules could do we must first figure out how much reacting each molecule can do. A logical starting place is to take the same number of molecules of each chemical--how could we do that? By taking one GMW of each chemical we know that we have eactly Avogadro's number of molecules. Now if the individual molecules did the exact minimum amount of reacting we could stop right there. But if each molecule could react twice, we would only need half a GMW for the same reaction. If each molecule could react three times, we would only need a third of a GMW, and in general, to get a consistant amount of reaction we need only take a GMW divided by the number of times each molecule can react. All that is left is to find a way to figure how much reacting each molecule can do. We do this with valence, a number assigned to each element in a molecule to describe that element's capacity to react. These are conveniently listed in a table. Some elements have plus valences while others have minus ones--depending on how they react (a plus reacts with a minus but not usually another plus). In any molecule, the elements have already reacted with each other, so the sum of the plus valences equals the sum of the minus valences and the molecule as a whole has a zero total. Since the sum of the plusses is the same as the sum of the minuses we need only consider one or the other for our index of the reacting strength within the molecule--we shall take the plusses, and call it the Total Positive Ionic Valence (abbreviated TPIV). For our purposes in this course we will further limit the TPIV to simply referring to the first element listed in a compound, and to calculate the TPIV of any molecule, we will take its first element's valence and multiply by the number of that element we have. For instance, K_2SO_4 has a TPIV of 2, since our chart shows the valence of K to be +1 and we have two of them. There are two additional situations we might encounter. First, you will notice that some elements have more than one valence value listed. In these cases, we recall that the sum of the plus valences must equal the sum of the minus ones, so by going through the remainder of the molecule we can figure out what the first element must be (such as $CuCl_2$, since Cl is -1 and we have two making -2, Cu must have been +2). Our second additional concern is when we have a radical in the compound--a combination of elements which act as a group and are usually enclosed in parenthesis. You will find all radicals you might encounter in this course listed on a table. Notice that with one exception all of the radicals have minus valences. This means they will not matter except when more than one valence is given for the first element. The remaining radical, ammonia or NH_4 has a +1 valence, so in dealing with any compound starting with N you will want to check to see if it is the ammonia radical (for instance $(NH_4)_2SO_4$, the first 'element' is NH_4 has a +1 valence, and since there are two the TPIV is 2). Remember that regardless of the number or chemical the TPIV is always a whole number and has no units of report.

We can now return to our constant reacting strength where we said to divide the GMW by the reacting strength of each molecule and simplify this to GMW/TPIV. This equivalent reacting amount of a chemical is defined as a Gram Equivalent Weight, abbrevaited GEW, and sometimes called by the shorter name Equivalent, abbreviated Eq. To find the GEW of H_2SO_4 we would find that its GMW is 98 g. and its TPIV is 2, so its GEW is 49 g.

The GEW of any compound has the same reacting strength as the GEW of any other compound. If we took these amounts and dissolved them in a liter of water, we would have equivalently reacting solutions, and would have found the concentration unit we set out to find. Therefore we define normality as the number of GEW's of solute per liter of total solution. Meeting our continual problem of how much solute is needed to prepare a given volume of a given normality is very similar to the way we solved molarity problems. For example: "How much H_2SO_4 is needed to prepare 2 dl of 6 N H_2SO_4 solution?" Our formula to solve this sort of problem is:

$$g = (N)(GEW)(L)$$

where N is the normality given (6N), GEW the calculated gram equivalent weight of the compound (49 g) and L the desired volume of solution (2 dl). After writing the formula and plugging in the given values, we once more stop to check whether the volume is in liters as the formula requires. When it is not, we convert it (2 dl = .2 L). Finally we do the multiplication and express our answer in grams (14.7 g).

With the addition of normality, we now have three choices of methods of expressing concentrations depending on how we intend to use the solution. This is the last major type of concentration unit we will consider in this course, although in the next hour we will consider some interesting variations of normality.

Molarity is useful for measuring some concentrations and normality for others. Occasionally, we will have a solution which has a labeled concentration in molarity but where for a reaction we need to know its normality. For this, we need to know how to convert between the two units. A second desire we might have is to make life a little easier--in the clinical laboratory we spend a great deal of time with very dilute body fluids, such as .005 N K^+ or .1 N Cl^-. This frequent encounter with small numbers inspires us to create an altered form of normality for very dilute solutions.

We can easily see that while molarity is adequate for some cases normality is needed for others. Fortunately, conversions betwen the two concentration units are not difficult. Our conversion formula is:

$$N = (M)(TPIV)$$

We need only plug in the concentration given, figure the TPIV, and solve the equation. For example, 3 M H_2SO_4 = ____ N? Since the TPIV = 2, the N = 6 N. If we were going the reverse direction (we were given the 6 N and asked for M), we could use the same equation and solve for M or we could use:

$$M = (N) / TPIV$$

Either way we would get 3 M. The two most common errors made by students on these problems are doing them backwards or having the wrong unit in the answer. Therefore it is not a bad idea to check two things on completing a problem. First, are the answer units consistent with what the question asks? Second, compare the numbers written by N and M in your work. Is the number by the M bigger? If so, it was done backwards and you need to try it again. This is easier to remember if you think of M standing for small or Mini (as in Mini-skirt).

We turn our attention now to the very dilute solutions, but first a quick review of normality. TPIV is the valence of the first element times the number of atoms of that element in the molecule. A GEW is a GMW/TPIV. Normality is the number of GEW's of solute per liter of total solution. When we turn to very dilute solutions with very small amounts of solute, we will turn to smaller units. Fortunately, the metric system allows us to do that by simply adding a prefix. What is the prefix meaning 1/1000? Milli or m. If we tacked that on to Normality we would get mN. for 1/1000 of the concentration. Unfortunately, you will never see this unit written this way. Instead we recall that N = GEW/L and that a synonym for GEW is Equivalent, abbreviated Eq. Thus we could write N = Eq/L, so when we took 1/1000 we would have mN = mEq/L, and this is the way the unit is written. Remember that even though it may look strange, mEq/L is nothing but mN or .001 N.

Problem solving with mEq/L (milliequivalent solutions) turns out to be very simple. We are already familiar with metric conversions from milli to the unit line (mg to g, ml to L, etc.), all accomplished by moving the decimal three to the left. So when we have mEq/L in a problem, which is nothing but mN, all we need to do is move the decimal three to the left and rewrite the problem with normality. For example, "How much NaCl is needed to make 2 liters of 5 mEq/L NaCl solution?" Simply converts to "How much NaCl is needed to make 2 liters of .005 N NaCl solution?" We then can proceed to work the problem just as we worked all of the previous normality problems.

We have now considered all of the concentration units (and any other types of units) that are used in this course. We can describe solutions in terms of how they were made (percent), number of solute molecules (molarity), how strongly they react (normality), and even for very dilute solutions in numbers of reasonable size (milliequivalent). Next we will consider what happens to these solutions when we use them in one way or another.

We have learned to describe solutions fully; it is now time to consider what we can do with the solutions. In general, we could use the solution in a procedure, or we could use the solution to make another solution. It is these uses that we will consider now.

When we use a solution to make a weaker solution, we are making a dilution, defined as the addition of solvent to a solution (which decreases the concentration of the solute). Frequently we are concerned with the degree of the dilution; that is, how much weakening of the solution has taken place. This is characterized by the dilution factor, defined as the ratio of the original volume to the final total volume, and expressed as "1:______." To find this takes two steps. First we write the original volume and a colon (for a dilution of 3 ml serum and 4 ml water, asked for the D.F. of the serum, we would start 3:___). After the colon we write the total amount of the solution (3:7). Our second step is to reduce the ratio to 1: ____ by dividing the first number into the second (1:2.33). Notice there are no units in the answer for the dilution factor. Also, since there may be several steps in a dilution it is important to notice which dilution factor is being asked for--one solution may have several different ones.

In many cases our dilution is simply to take some concentration of a solution and make a weaker solution. Notice when we do this, we are adding solvent only, not solute. The amount of solute in the solution remains unchanged. But since all of our concentrations are in terms of the amount of solute in a certain volume of solution, the amount of solute equals the product of the concentration and the volume of the solution. Since the amount does not change, the product before the dilution must equal the product afterward, so

$$C_1V_1 = C_2V_2$$

where C is concentration, V is volume, and the subscripts refer to the different solutions. To work problems using this formula, such as "How much 4 M HCl is needed to prepare 2 ml of 2 M HCl?" we would first write the formula, then plug in corresponding values on the same side of the equation. It may help to notice that in most of these problems the word 'of' acts as a bridge to tell you which concentration and volume go together on the same side (above, "of" connects 2 ml and 2 M). We will need to put in a variable like "x" for the unknown value. At this point we stop and compare--are the two concentrations in the same units? If not, we will need to convert one. Also at this point we can tell the unit of our answer, since it must have the same unit as its corresponding type of unit on the other side. You will avoid some careless errors if at this time you write the units of the answer next to the variable. (4 M) (x ml) = (2 M)(2 ml). Now we need only solve the equation; being careful to put the right units in our answer (1 ml). Notice that the equation has no specified units. It does not matter what units are used for concentration or for volume as long as the same units are used for both C's and the same units for both V's.

We also may wish to react specific solutions together, usually an acid and a base. This is called a titration, and is defined as determining an unknown concentration by reacting the solution exactly with a known concentration solution. Since the two react exactly, the same reacting strength of solute must have been used up in each case. But Normality is defined as number of equivalents per liter, so the product of normality and the volume of the solution would equal the number of reacting strengths used up. Since this is the same for both solutions, we again get that the products of concentration and volume for the two solutions must be equal:

$$C_1V_1 = C_2V_2$$

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A typical problem of this sort reads "It took 2 dl of 3 N HCl to neutralize 4 dl of an unknown base. What is the concentration of the base?" We work these exactly like dilution problems, still using the word 'of' to tell us which concentration goes with which volume, and still getting the unit of our answer from the corresponding unit of the same type.

This completes the basic material of our general chemistry review. We now have the information necessary to handle the bulk of the calculation problems encountered in the clinical chemistry laboratory.

Now that we have covered all the different kinds of problems encountered in the general chemistry review, there is ample room for confusion in deciding which formula is used with which problem. We will cover some general rules to aid in that decision, and practice working various types of problems.

The first major type of problem is the "think" problem. They are called this because the only thing that takes place is calculations--no action at all. They are characterized by their use of words such as 'convert', 'what is', 'express', etc. Such problems will always be regular metric conversions, temperature conversions, or conversions between normality and molarity.

The larger group of problems are the "action" problems, where something is to be done and you must figure out either how to do it or what happened. These roblems have action words like "make," "prepare," "how much is needed," "dilute," "titrate," "neutralize," etc. To decide which formula applies try the following steps. First, be able to recognize a solution in a problem. To be a solution, it must have a quantifier (4), a concentration unit (M) and a chemical (HCl) (referring to 4 M HCl). After you have decided that it is an action problem, count the solutions in it. If there are two, you will be using $C_1V_1 = C_2V_2$. If ther is only one solution, the formula will be among the first three we learned:

$$g = (g/dl)(DL)$$

$$g = (M)(GMW)(L)$$

$$g = (N)(GEW)(L)$$

You decide which of these by using the one that includes the concentration unit given for the solution in the problem. Notice that if that unit were mEq/L, you must first do the conversion to normality.

The examination that you had today should give you a good idea of where you are with respect to the crucial examination. If your work indicated that you might encounter difficulty passing the crucial examination, your counselor or another instructor will approach you about how you might improve your skill level.

SP4 Richard L. Holmes

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-205-1
LABORATORY SCIENCES DIVISION 116
CHEMISTRY BRANCH

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General Chemistry Equations

A. Metric System Prefixes

Prefix	Abbreviation	Scientific Notation	Numerical Value
Mega-	M	10^6	1,000,000
Kilo-	k	10^3	1,000
Hecto-	h	10^2	100
Deka-	da	10^1	10
----	--	10^0	1
Deci-	d	10^{-1}	0.1
Centi-	c	10^{-2}	0.01
Milli-	m	10^{-3}	0.001
Micro-	u	10^{-6}	0.000001
Nano-	n	10^{-9}	0.000000001

B. Solution Formulae

1. $g = (g/dl)(dl)$, $mg = (mg/dl)(dl)$, $ml = (ml/dl)(dl)$

2. $g = (M)(GMW)(L)$

3. $g = (N)(GEW)(L)$

4. $C_1V_1 = C_2V_2$

C. Conversion Formulae

1. $^{o}F = (^{o}C \times 1.8) + 32$

2. $^{o}C = \frac{^{o}F - 32}{1.8}$

3. $N = (M)(TPIV)$, $M = \frac{N}{TPIV}$

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*This mimeo supersedes M 08-280-205-1, 123.

SP/4 Richard L. Holmes

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-205-2
LABORATORY SCIENCES DIVISION 076
CHEMISTRY BRANCH

Homework: Metric Conversions

1. 4,250 ml = __________ L
2. 2,751 L = __________ ml
3. 15 g = __________ mg
4. 1530 mg = __________ g
5. 0.06 g = __________ mg
6. 237 dl = __________ L
7. 367 cg = __________ mg
8. 42 m = __________ mm
9. 2376 L = __________ dl
10. 74 µl = __________ ml
11. 467 M = __________ hm
12. 0.064 g = __________ cg
13. 4,632 nl = __________ dl
14. 4 L = __________ dl
15. 40 mg = __________ g
16. 3,274,639 µg = __________ g
17. 73 ml = __________ L
18. 4.67 L = __________ dl
19. 7.352 hg = __________ dg
20. 6.39 dl = __________ L
21. 95 mg = __________ g
22. 0.00003 L = __________ ml
23. 2.645 dl = __________ L
24. 6493.64 ml = __________ L
25. 5.003 L = __________ ml
26. 37 dl = __________ L
27. 629.4 L = __________ dl
28. 8.47 mm = __________ µm
29. 0.006 dl = __________ D
30. 0.006 ml = __________ dl
31. 6.235 mg = __________ cg
32. 372 dam = __________ m
33. 0.001 g = __________ cg
34. 9.93 hg = __________ dag
35. 0.04 ml = __________ dl
36. 2,364 ml = __________ µl
37. 8,659.1 mg = __________ g
38. 0.004 L = __________ dl
39. 36 L = __________ ml
40. 51.7 dl = __________ L

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*This mimeo supersedes M 08-280-205-2, 063.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-205-3
LABORATORY SCIENCES DIVISION 103
CHEMISTRY BRANCH

Chemical Data of Common Elements and Radicals

NAME	SYMBOL	APPROX. ATOMIC WEIGHT	VALENCE
Aluminum	Al	27	+3
Antimony	Sb	122	+3
Arsenic	As	75	+3
Barium	Ba	137	+2
Bismuth	Bi	209	+3
Bromine	Br	80	-1
Calcium	Ca	40	+2
Carbon	C	12	+4
Chlorine	Cl	35.5	-1
Chromium	Cr	52	+3, +6
Copper	Cu	64	+1, +2
Fluorine	F	19	-1
Gold	Au	197	+3
Hydrogen	H	1	+1
Iodine	I	127	-1
Iron	Fe	56	+2, +3
Lead	Pb	207	+2, +4
Lithium	Li	7	+1
Magnesium	Mg	24	+2
Manganese	Mn	55	+2
Molybdenum	Mo	96	+6
Mercury	Hg	201	+1, +2
Nickel	Ni	59	+2, +3
Nitrogen	N	14	+3, -3
Oxygen	O	16	-2
Phosphorus	P	31	+5
Potassium	K	39	+1
Silicon	Si	28	+4
Silver	Ag	108	+1
Sodium	Na	23	+1
Strontium	Sr	88	+2
Sulfur	S	32	-2, +6
Tin	Sn	119	+2, +4
Tungsten	W	184	+6
Zinc	Zn	65	+2

POSITIVE RADICALS

NAME	SYMBOL	VALENCE
Ammonium	NH_4	+1

NEGATIVE RADICALS

NAME	SYMBOL	VALENCE
Acetate	$C_2H_3O_2$	-1
Bicarbonate	HCO_3	-1
Bisulfate	HSO_4	-1
Carbonate	CO_3	-2
Chlorate	ClO_3	-1
Dichromate	Cr_2O_7	-2
Hydroxide	OH	-1
Hypochlorite	ClO	-1
Nitrate	NO_3	-1
Nitrite	NO_2	-1
Oxalate	C_2O_4	-2
Permanganate	MnO_4	-1
Phosphate	PO_4	-3
Sulfate	SO_4	-2
Sulfite	SO_3	-2
Tungstate	WO_4	-2

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*This mimeo supersedes M 08-280-205-4, 022.

SP4 Richard L. Holmes

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY M 08-280-205-4
LABORATORY SCIENCES DIVISION 116
CHEMISTRY BRANCH

Basic Algebra Review

In a simple equation, we know both sides are equal, such as: $2 + 4 = 6$

In an algebra equation, one of the numbers has been replaced with a letter:
$2 + x = 6$

When we talk about solving an algebra equation, we mean finding out what number was replaced. Since the 4 was replaced above, our answer would be: $x = 4$

The letter is nothing more than a substitute we use for a number until we can find out what the number is.

There are three basic types of problems--those in which we add or subtract using a letter, those in which we multiply or divide using a letter, and those in which we do both things. Some examples of the first type are:

(a) $X + 3 = 5$ (b) $X - 4 = 8$

To solve such problems or any other algebra problems, we must be careful to always do the same thing on both sides of the equal sign. For instance, if +4 is added to the left, it must also be added to the right. To solve the problem of the type above, we do the opposite of whatever is being done to the letter. For example, in (a) 3 is added to the letter, so we subtract 3:

(a) $X + 3 = 5$
$X + 3 - 3 = 5 - 3$

(b) $X - 4 = 8$
$X - 4 + 4 = 8 + 4$

Notice that whatever we did, we did it to both sides. What's more, if we add and subtract the same number (+ 3 - 3), we have not changed the basic number at all, so:

(a) $X + 3 = 5$
$X + 3 - 3 = 5 - 3$
$X = 5 - 3$
$X = 2$

(b) $X - 4 = 8$
$X - 4 + 4 = 8 + 4$
$X = 8 + 4$
$X = 12$

Time out to practice a few problems:

1. $y + 3 = 7$
2. $c - 4 = 8$
3. $X - 5 = 9$
4. $V + 2 = 8$
5. $k - 4 = 1$
6. $X + 3 = 4$

Multiplication/division problems are done the same way, except when we do the opposite of whatever was being done to the letter, we are multiplying or dividi

(c) $3X = 5$
$3X + 3 = 5 + 3$

$X = 1.67$

(d) $\frac{y}{3} = 6$
$\frac{y}{3} \times 3 = 6 \times 3$
$y = 18$

(e) $4 = k + 3$
$4 \times 3 = k + 3 \times 3$
$12 = k$

Notice that division can be written two ways, $\frac{k}{3}$ or $k \div 3$. If a number and letter are written together (3X) it means multiplication (3 x X, or 3 times X). More practice:

7. $2X = 8$
8. $5 = \frac{Z}{3}$
9. $4 = 5X$
10. $Y \div 7 = 3$
11. $6K = 12$
12. $14 = \frac{V}{2}$

Sometimes both operations are involved:

(f) $3X + 4 = 12$

(g) $\frac{k}{2} - 6 = 3$

First we use the method of the addition problems and then the method of the multiplication:

(f)
$$3X + 4 = 12$$
$$3X + 4 - 4 = 12 - 4$$
$$3X = 8$$
$$3X \div 3 = 8 \div 3$$
$$X = 2.67$$

(g)
$$\frac{K}{2} - 6 = 3$$
$$\frac{K}{2} - 6 + 6 = 3 + 6$$
$$\frac{K}{2} = 9$$
$$\frac{K}{2} \times 2 = 9 \times 2$$
$$K = 18$$

Final practice:

13. $2X + 4 = 10$
14. $\frac{K}{3} - 2 = 2$
15. $6 = 8 + \frac{K}{4}$
16. $(K \div 6) + 2 = 8$
17. $3X - 4 = 8$
18. $6 = 4X + 2$

Review:

19. $Y + 5 = 13$

20. $4X = 16$

21. $3Y - 2 = 13$

22. $\frac{a}{3} - 1 = 2$

2

23. $12 = (X \div 4) + 7$

24. $14 = 3X$

25. $9 = 2K - 11$

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-210
LABORATORY SCIENCES DIVISION 034
CHEMISTRY BRANCH

Spectrophotometry (7 hours)

COURSES PRESENTED TO: 311-92B20, Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES:

Lee, L.W. Elementary Principles of Laboratory Instruments. 2d ed. Saint Louis: C.V. Mosby Co., 1970, chap 2.
Tietz, N.W. Fundamentals of Clinical Chemistry. 1st ed. Philadelphia: W. B. Saunders Co., 1970, pp 87-98.

STUDY ASSIGNMENT: After class review M 08-280-210-1, -2, and -3.

STUDENT UNIFORM AND EQUIPMENT: Slide rule and laboratory notebook.

TOOLS, EQUIPMENT, AND MATERIALS: M 08-280-210-1 issued at the beginning of the first hour. M 08-280-210-2, and -3 issued at the beginning of the third hour. M 08-280-210-4 to be used at the instructor's discretion for remedial or additional teaching.

PERSONNEL: One (1) instructor (MOS 92B4H) 1st, 2nd, and 7th hours; eight (8) instructors (MOS 92B4H) 3rd thru 6th hours.

INSTRUCTIONAL AIDS: Chalk, chalkboard, and eraser; standard laboratory reagents and equipment; Videotapes numbers 275 and 362 (see Annex D) and receivers.

TROOP REQUIREMENTS: Not applicable

TRANSPORTATION REQUIREMENTS: Not applicable

TYPE OF INSTRUCTION: Lecture (1st and 2d hours); practical exercise (3rd -6th hours); examination (7th hour).

FIRST AND SECOND HOURS

NOTE: Issue M 08-280-210-1, one per student.

I. INTRODUCTION (5 min)

A. Opening Statement: The primary aim of most work in clinical chemistry is the determination of the concentration of a particular substance as glucose or urea in the blood of a patient. Since we are dealing with the concentration of very minute molecules, we cannot use direct counting techniques such as used in hematology. Most clinical chemistry procedures involve the production of colored solutions by the reaction of reagents and the substance to be tested. The amount of color produced, which is proportional to the substance tested for, is then measured. With this information the concentration of the substance being tested for can be calculated.

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*This lesson plan supersedes LP 08-280-220, 081; 08-280-225, 081; and 08-280-230, 081.

B. Objectives

1. List the six main parts of a spectrophotometer and their functions.

2. Calculate sample concentrations using Beer's law.

3. Define selective absorption.

4. Identify the need for a standard curve in verifying Beer's law and calculating unknown concentrations.

5. Determine the absorbance of unknown samples by using a spectrophotometer, to include calibrating, zeroing, and blanking of the instrument.

C. Class Procedure and Lesson Tie-in

Almost half of the chemistry procedures used in this course involve the use of a spectrophotometer and Beer's law to calculate unknown sample concentrations.

II. EXPLANATION (75 min)

A. Colorimetric Principles

NOTE: The following is the basis for many clinical chemistry determinations.

1. Formation of colored solution

2. Amount of color proportional to sample concentration

3. Colorimetric comparison of standard and unknowns

4. Calculation by ratio and proportion

B. Terminology

1. Wavelength

   a. Definition - distance from peak to peak

b. Symbol - lambda (λ)

c. Units - nanometer (nm)

2. Spectrum

a. Visible

(1) Range 400 - 700 nm

(2) Colors

NOTE: Students are not required to memorize colors and their ranges.

(a)	Violet	400 - 425 nm
(b)	Blue	425 - 490 nm
(c)	Green	490 - 575 nm
(d)	Yellow	575 - 585 nm
(e)	Orange	585 - 650 nm
(f)	Red	650 - 700 nm

b. Non-visible

NOTE: Some clinical determinations use ultraviolet or infrared light, but most use visible light.

(1) Ultraviolet (UV) (Below 400 nm)

(2) Infrared (IR) (above 700 nm)

C. Selective Absorption

1. Definition - the ability of a substance to absorb certain wavelengths of light while transmitting others.

2. Explanation

QUESTION: Why does a blue solution appear blue to the human eye?

ANSWER: A blue solution transmits all or most of the blue wavelengths of light while most or all of the other wavelengths are absorbed.

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D. Spectrophotometer Parts

1. Electronic power source

   a. Transformer

   b. Battery

2. Light source

   a. Incandescent lamp (visible light)

   b. Hydrogen lamp (ultraviolet light)

3. Monochromator

   a. Diffraction grating - spectrum production

NOTE: Some spectrophotometers may have a prism.

   b. Exit slit - wavelength selection

4. Cuvet - holds solution

5. Photodetector - converts light energy into electrical energy

6. Read out device

   a. Galvanometer - converts electrical energy into mechanical energy

   b. Scale panel

      (1) Percent transmittance scale

      (2) Absorbance scale

E. Beer's Law

1., Light absorption proportional to concentration

2. Light absorption proportional to length of light path

NOTE: See ANNEX A for demonstration of light path and concentration effects on a solution's absorbance.

QUESTION: If the concentration of a color producing substance in a solution increases, does the absorbance increase or decrease?

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ANSWER: The absorbance of the solution also increases.

3. Algebraic expression

NOTE: Beer's law is a mathematical statement of the principle of selective absorption: the absorbance of monochromatic light by a solution is equal to the product of the concentration of the light absorbing particles, times the distance the light travels through the solution, times a constant.

$$A = C \times b \times a$$

A = amount of light absorbed

C = concentration of the light absorbing particles

b = length of the light path

a = constant for the light absorbing characteristics of a substance

QUESTION: What determines the length of the light path?

ANSWER: The internal diameter of the cuvet used determines the light path length.

4. Calculations

NOTE: Results are obtained by mathematically comparing the absorbance of an unknown sample with the absorbance of a standard sample.

a. Formula derivation

NOTE: See Annex B. Students are not to memorize derivation.

b. Formula forms

$$(1)\quad \frac{A_s}{C_s} = \frac{A_u}{C_u}$$

NOTE: This formula can be most easily used with a slide rule as a ratio and proportion operation.

$$(2)\quad C_u = \frac{A_u}{A_s} \times C_s$$

NOTE: This is an alternate form for doing the calculations longhand.

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QUESTION: What are the concentrations for the following glucose unknowns?

	A	C
Standard	0.26	200 mg/dl
Unknown 1	0.13	
2	0.11	
3	0.37	
4	0.49	

ANSWER: $\frac{A_s}{C_s} = \frac{A_u}{C_u}$

$$\frac{0.26}{200\text{ mg/dl}} = \frac{0.13}{100\text{ mg/dl}} = \frac{0.11}{85\text{ mg/dl}} = \frac{0.37}{284\text{ mg/dl}} = \frac{0.49}{377\text{ mg/dl}}$$

F. Standard Curve

1. Establishment of curve

   a. Set of standard samples — known concentrations

   b. Perform test

   c. Record absorbancies

   d. Plot concentration versus absorbancies

NOTE: Draw Annex C.

2. Uses of curve

   a. Check validity of Beer's law

      (1) Straight line plot -- absorbancies follow Beer's law

      (2) Curvilinear plot -- absorbancies do not follow Beer's law

   b. Calibration curve -- unknown concentrations read directly from curve

G. Questions from Students

III. SUMMARY (10 min)

A. Review of Main Points

1. Colorimetric principles
2. Terminology
3. Selective absorption
4. Spectrophotometer parts
5. Beer's law
6. Standard curve

B. Closing Statement: Because a spectrophotometer is involved in numerous clinical chemistry procedures, it is important for the medical laboratory specialist to be familiar with the principles, parts, and calculations involved with the use of a spectrophotometer.

THIRD THROUGH SIXTH HOURS

IV. APPLICATION (170 min)

NOTE: Hand out M 08-280-210-2, -3, one of each per student.

A. Videotapes (25 min) (see Annex D)

NOTE: Videotapes are used to supplement instructions for the practical exercise.

1. Introduction

   a. Introduction to Spectrophotometry (11:36)

      (1) Points to be observed

         (a) The use of selective absorption in the Clinical laboratory

         (b) Parts of the spectrophotometer

         (c) Procedure for checking calibration

         (d) Procedure for reading unknown absorbancies

      (2) Showing of videotape No. 275

   b. Review of Spectrophotometry (8:30 min)

- (1) Points to be observed
  - (a) Use of Coleman Jr. 6-A
  - (b) Use of Coleman Jr. II
  - (c) Use of Bausch and Lomb Spectronic 20
- (2) Showing of videotape No. 362

2. Discussion and Questions

B. Instructors' Activity

1. Explain blanking and zeroing of spectrophotometers
2. Monitor use of spectrophotometers
3. Check results and conclusions for accuracy
4. Ensure proper clean-up of spectrophotometers and area

C. Students' Activity

1. Zero and blank spectrophotometers
2. Calibrate spectrophotometers
3. Perform practical exercise
4. Clean equipment and laboratory area

NOTE: Procedures for above operations are in M 08-280-210-2.

V. **REVIEW** (10 min)

A. Questions From Students

B. Review of Main Points

1. Sources of error
2. Precautions

C. Closing Statement: Correct use of a spectrophotometer, and the correct calculation of unknown concentrations will be the final steps in many of our procedures. With the material just presented, you should be able to perform these steps completely and accurately.

SEVENTH HOUR

VI. EXAMINATION (45 min)

A. Directions to Students

1. Answers

    a. Put on quiz paper.

    b. Show all work

2. Utilize

    a. Formula sheet

    b. Slide rule

B. Instructors' Activities

1. Issue quiz, one per student

2. Monitor quiz

3. Collect completed quiz sheets

4. Grade completed quiz sheets

C. Students' Activities

1. Put individual identification on quiz sheet

2. Answer quiz questions as appropriate

3. Submit completed quiz for grading

D. Critique

1. Review quiz

2. Reteach quiz

3. Questions from students

ANNEX A
INSTRUCTIONAL AIDS

Demonstration

The importance of the concentration of a light absorbing substance and the length of the light path can be shown by filling two volumetric flasks with solutions of Evans blue dye or any other colored solution. Use half as much Evans blue in making up the second solution as in the first. The effect of concentration on absorption can be shown by comparing the intensity of the color between the two flasks. The effect of light path can be demonstrated by observing the difference in color intensity between the neck of the flask and its wider base.

NOTE: Use demonstration in part IVE2.

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Derivation of Modified Beer's Law Formula

Beer's Law States:

$A_s = C_s \times b_s \times a_s$ for the standard solution

$A_u = C_u \times b_u \times a_u$ for the unknown solution

If the light absorbing substance is the same

$$a_s = a_u$$

If the same sized cuvets are used

$$b_s = b_u$$

Therefore:

$$a_s \times b_s = a_u \times b_u$$

By rearranging Beer's Law

$$\frac{A_s}{C_s} = a_s \times b_s \quad \text{and} \quad \frac{A_u}{C_u} = a_u \times b_u$$

From above

$$\frac{A_s}{C_s} = a_s \times b_s = a_u \times b_u = \frac{A_u}{C_u}$$

Simplifying

$$\frac{A_s}{C_s} = \frac{A_u}{C_u} \quad \text{or} \quad C_u = \frac{A_u}{A_s} \times C_s$$

NOTE: Used in part IIE4a.

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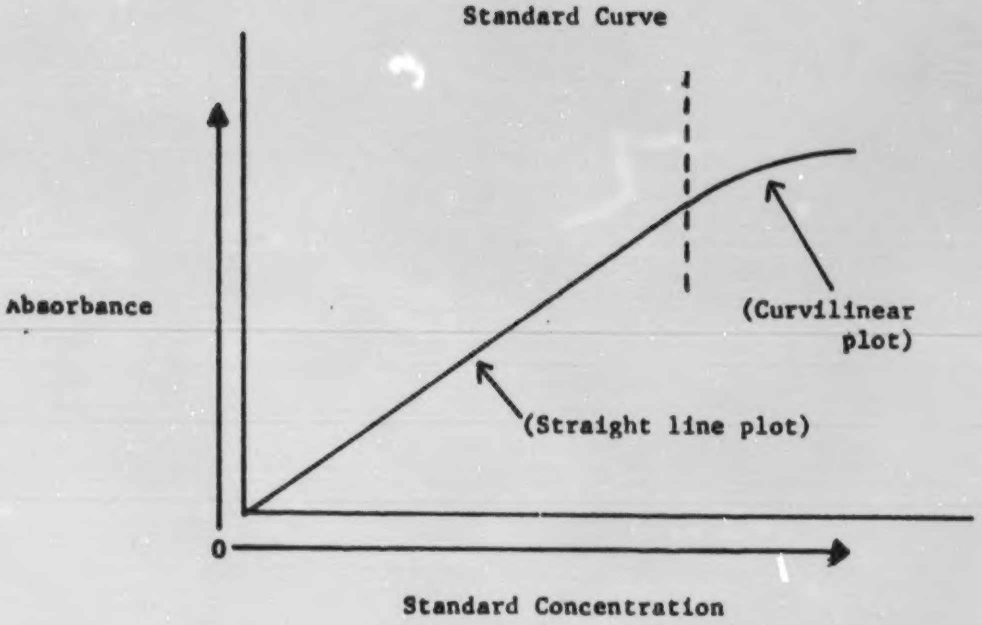
Chalkboard Drawing



NOTE: Used in part IIF1d.

ANNEX D
INSTRUCTIONAL AIDS

Videotape

VT No. 275, 11:36 minutes. Introduction to Spectrophotometry.

The spectrophotometer is illustrated as an instrument that converts white light into spectral colors, using one of the colors produced to measure the light absorbance of a solution. The function of the parts of a spectrophotometer are shown. The steps involved in the use of a spectrophotometer are demonstrated including the use of a didymium filter, a reagent blank, and a sample.

NOTE: This videotape is shown in part IV prior to the students' practical exercise.

VT No. 362, 8:30 minutes. Review of Spectrophotometry.

The step by step use of three common spectrophotometer is demonstrated: the Coleman Junior 6A, the Coleman II 6/12, and the Bausch and Lomb Spectronic 20. The need for zeroing and blanking a spectrophotometer is explained.

NOTE: This videotape is shown in part IV prior to the students' practical exercise.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *IM 08-280-210
LABORATORY SCIENCES DIVISION 034
CHEMISTRY BRANCH

Spectrophotometry

The primary aim of most work in clinical chemistry is the determination of the concentration of a particular substance as glucose of urea in the blood of a patient. Since we are dealing with the concentration of very minute molecules, we cannot use direct counting techniques such as used in hematology. Most clinical chemistry procedures involve the production of colored solutions by the reaction of reagents and the substance to be tested. The amount of color produced, which is proportional to the substance tested for, is then measured. With this information the concentration of the substance being tested for can be calculated.

Color formation is accomplished by following a procedure that has been designed to test for the substance whose determination is desired. The intensity of the color produced, which is proportional to the concentration of the substance in the sample, is measured by a spectrophotometer. Beer's law is then used to compare the standard and unknown absorbancies by ratio and proportion so that the concentrations can be calculated and reported to the medical officer.

Because numerous chemistry procedures depend on the production and measurement of color, it is important to become familiar with these concepts and methods. The basis of color is light, which travels in waves. Since different energies of light (different colors) are characterized by different sized waves, we measure their energy (color) in terms of their wavelength. One wavelength is the distance between adjacent peaks or adjacent valleys of a wave. The Greek letter lambda (λ) is used as the symbol for wavelength. The correct unit of measurement for wavelength is the nanometer (nm).

The wavelengths with which we are most often concerned are those that fall within the visible spectrum, that is, the wavelength from approximately 400 to 700 nanometers. Wavelengths between 400-425 nm are violet, 425-490 nm are blue, 490-575 nm are green, 575-585 nm are yellow, 585-650 nm are orange, and 650-700 nm are red. Some clinical determinations use non-visible light, such as ultraviolet (wavelengths lower than 400 nm) or infrared (wavelengths above 700 nm) light. But, most use visible light.

Why are substances the color they are? Why does one solution appear violet while another appears yellow? This phenomenon is due to a particular property of solutions called selective absorption. Selective absorption is the ability of a solution to absorb certain wavelengths of light while transmitting others. In the case of a violet solution, all the wavelengths of visible light are being absorbed except those in the violet range. These are being transmitted through the solution. Therefore, the solution appears violet in color.

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*This instructors manuscript supersedes IM 08-280-220, 081; 08-280-225, 081; and 08-280-230, 081.

After having reacted the substance which we wish to measure with reagents to produce a color, we use a spectrophotometer to measure the amount of light being absorbed by the solution, using the wavelength of light that is selectively absorbed to the greatest extent by the solution. Since the spectrophotometer is probably the most routinely used instrument in the clinical chemistry laboratory, it is necessary to know how to operate it and to understand the principles associated with its operation.

The initial requirement for a spectrophotometer is a power source, either from a wall socket or, in some cases, a battery. We must have a light source and some procedures use ultraviolet light. To produce this light a mercury vapor lamp or a deuterium lamp is used; however, to produce light in the visible range a tungsten filament lamp is used. Rather than simply using white light, photometric determinations utilize a specific wavelength or color of light. The wavelength used is the one at which optimum absorption takes place. In order to obtain a light of one color, (monochromatic) a spectrum must first be produced. A prism or a diffraction grating may be used for this purpose. A diffraction grating is normally used in spectrophotometers because it produces a truer, more even break-up of the light than does a prism. The spectrum that is produced strikes an exit slit which allows a monochromatic band of light to pass through while blocking the rest of the light. How is the wavelength which passes through the exit slit selected? The exit slit could be moved so that a different wavelength of light would pass through the opening or the angle of the prism or diffraction grating could be changed so that the spectral image would appear at a different position on the exit slit. In the Coleman spectrophotometers, the wavelength is selected by changing the angle of the exciter lamp in relation to the diffraction grating. This monochromatic light then passes through the solution in the cuvet. Some of the light is absorbed while the rest passes through the solution and strikes the photodetector. The photodetector converts this light energy into electrical energy which is then transmitted to the galvanometer converting it to mechanical energy. The galvanometer index moves along the galvanometer scale to give a reading. The galvanometer scale is divided into two different scales--one marked in units of percent transmittance and the other in units of absorbance. In most cases the absorbance scale is used. This is a logarithmic scale, read from right to left, and calibrated from 0 to 2. The absorbance of a solution that has been read from the galvanometer scale of a spectrophotometer is used in the calculation of the sample concentration for the test being run.

Beer's law is a mathematical equation that relates several factors which affect absorbancy. First, the amount of absorbance by a particular solution depends on the concentration of the light absorbing substance in solution. Absorption is directly proportional to concentration. That is, as the concentration of a colored solution increases, so does the absorption. A darker solution is more concentrated and absorbs more light. Secondly, the absorption of a solution depends on the length of the light path. The light path is the distance the light travels in passing through the solution. As the light path increases, the number of light absorbing particles that a beam of light will strike is increased and so is the absorption.

These concepts are tied together in Beer's law, which states that the absorbance of monochromatic light is equal to the product of the concentration of the light absorbing particles, times the length of the light path, times a constant. It is mathematically expressed as $A = C \times b \times a$. In this equation, A represents the light absorbance; C the concentration of the light-absorbing substance; b the light path length; and a the constant. In practice, the length of the light path is the internal diameter of the cuvet.

Colorimetric procedures are based on results obtained by mathematically comparing the absorbance of an unknown sample to the absorbance of a standard sample. Sample concentrations are calculated from a formula that is derived from Beer's law. The formula is most commonly seen in one of the following forms: $C_u = \frac{A_u}{A_s} \times C_s$ or $\frac{A_s}{C_s} = \frac{A_u}{C_u}$. The latter formula is expressed as a ratio and proportion and is the one most often used with the slide rule. The results that are reported to the medical officer are the sample concentrations (C_u) that have been calculated by using the formula. However, not all solutions follow Beer's law. In fact, it is not uncommon to find that Beer's law is not followed at high concentrations. Therefore, before a test is used in the clinical laboratory, the range over which Beer's law is valid must be established by using a standard curve. The test that is being checked is run on a series of standard samples; these are solutions that have been prepared containing varying, known concentrations. The absorbancies are read in a spectrophotometer. The standard curve is then drawn as the plot of the concentrations of the standard samples versus their respective absorbancies. If the graph is a straight line and passes through the origin, Beer's law is valid and can be used to calculate unknown concentrations. Where the graph is curvilinear, Beer's law is not valid and cannot be used in calculations.

In the material just covered, we have discussed the basic principles of many chemistry tests, common terminology used in spectrophotometry, selective absorption as the basis of color production, the parts and function of spectrophotometers in measuring the color, the use of Beer's law to calculate sample concentrations, and the use of a standard curve to check the validity of Beer's law.

Because a spectrophotometer is involved in numerous clinical chemistry procedures, it is important for the medical laboratory specialist to be familiar with the principles, parts, and calculations involved in the use of a spectrophotometer.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-210-2
LABORATORY SCIENCES DIVISION 073
CHEMISTRY BRANCH

Use of the Coleman Junior II Spectrophotometer

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*This mimeo supersedes M 08-280-220-3, 081.

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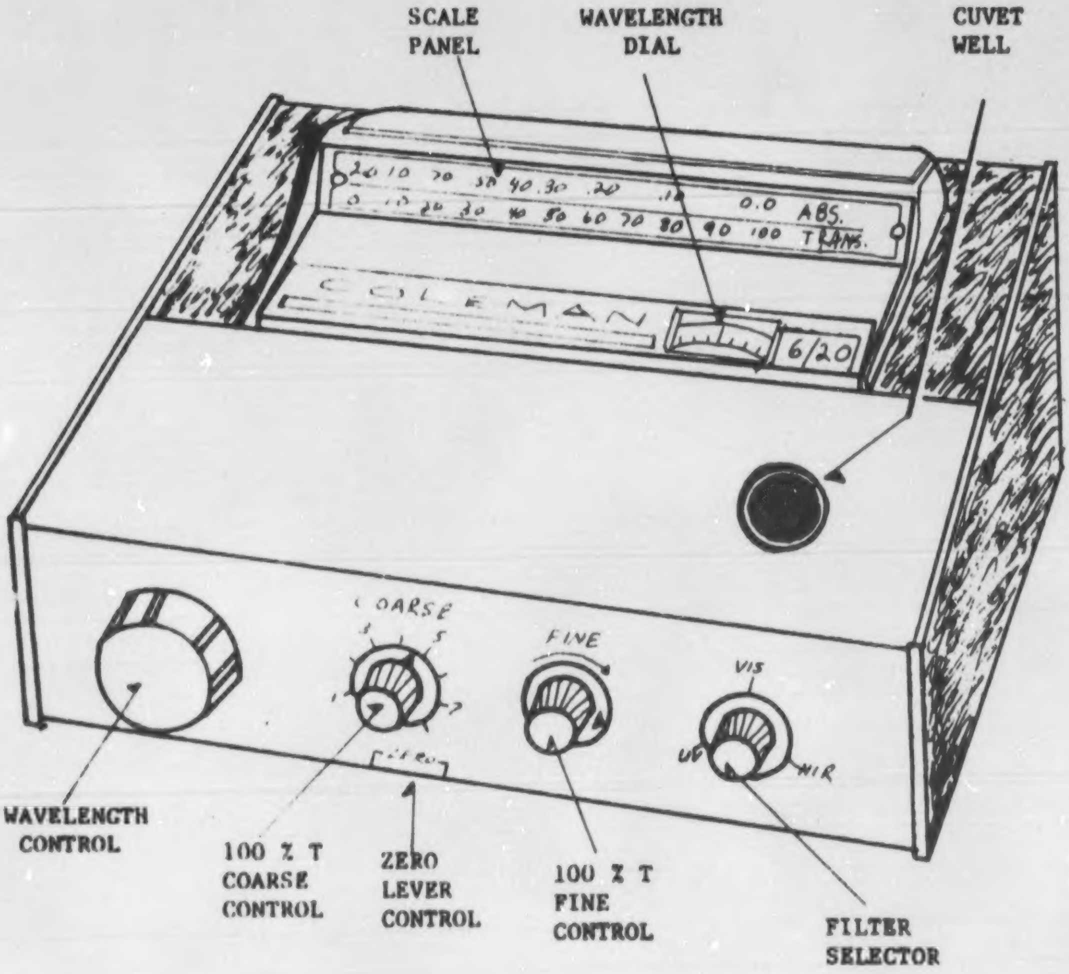
SCALE PANEL
WAVELENGTH DIAL
CUVET WELL
ABS.
TRANS.
COLEMAN
6/20
COARSE
FINE
VIS
UV
NIR
ZERO
WAVELENGTH CONTROL
100 % T COARSE CONTROL
ZERO LEVER CONTROL
100 % T FINE CONTROL
FILTER SELECTOR

Figure 1. Front view, Coleman Junior II spectrophotometer.

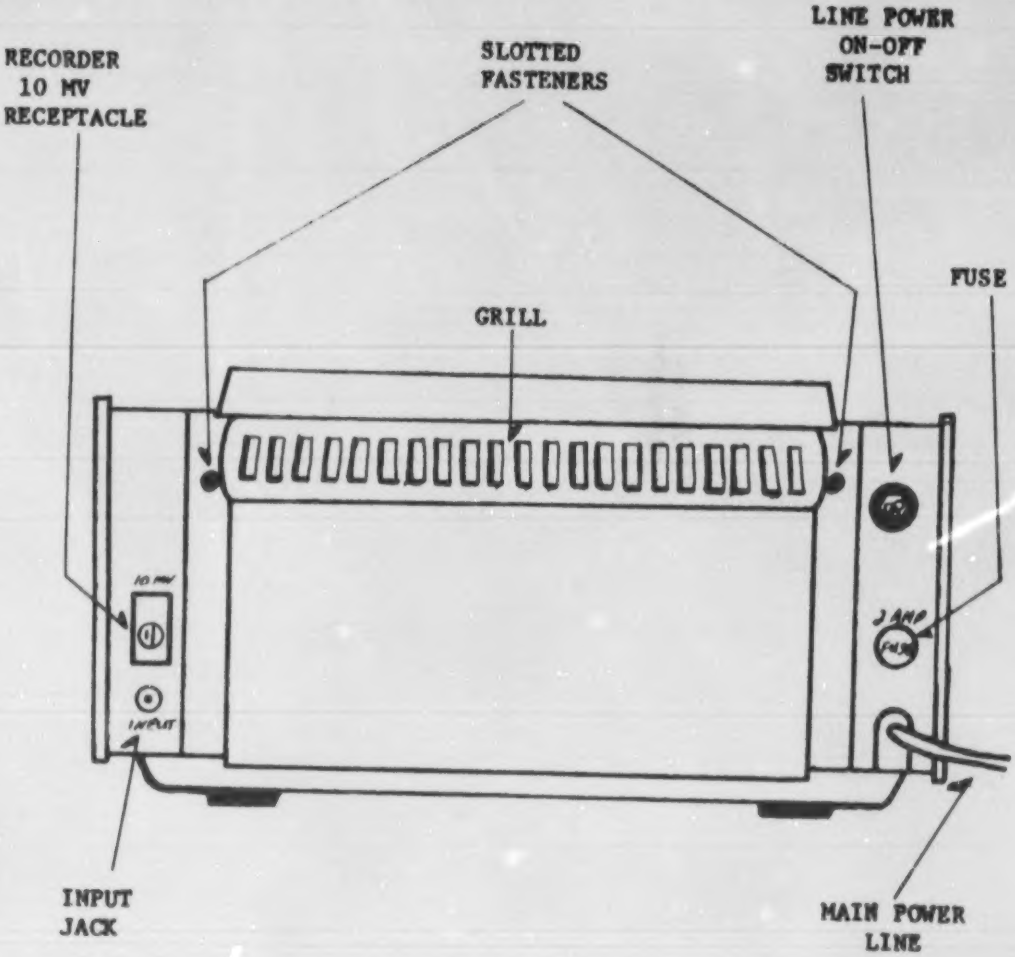
RECORDER
10 MV
RECEPTACLE
SLOTTED
FASTENERS
LINE POWER
ON-OFF
SWITCH
FUSE
GRILL
10 MV
INPUT
2 AMP
FUSE
INPUT
JACK
MAIN POWER
LINE

Figure 2. Rear view, Coleman Junior II spectrophotometer.

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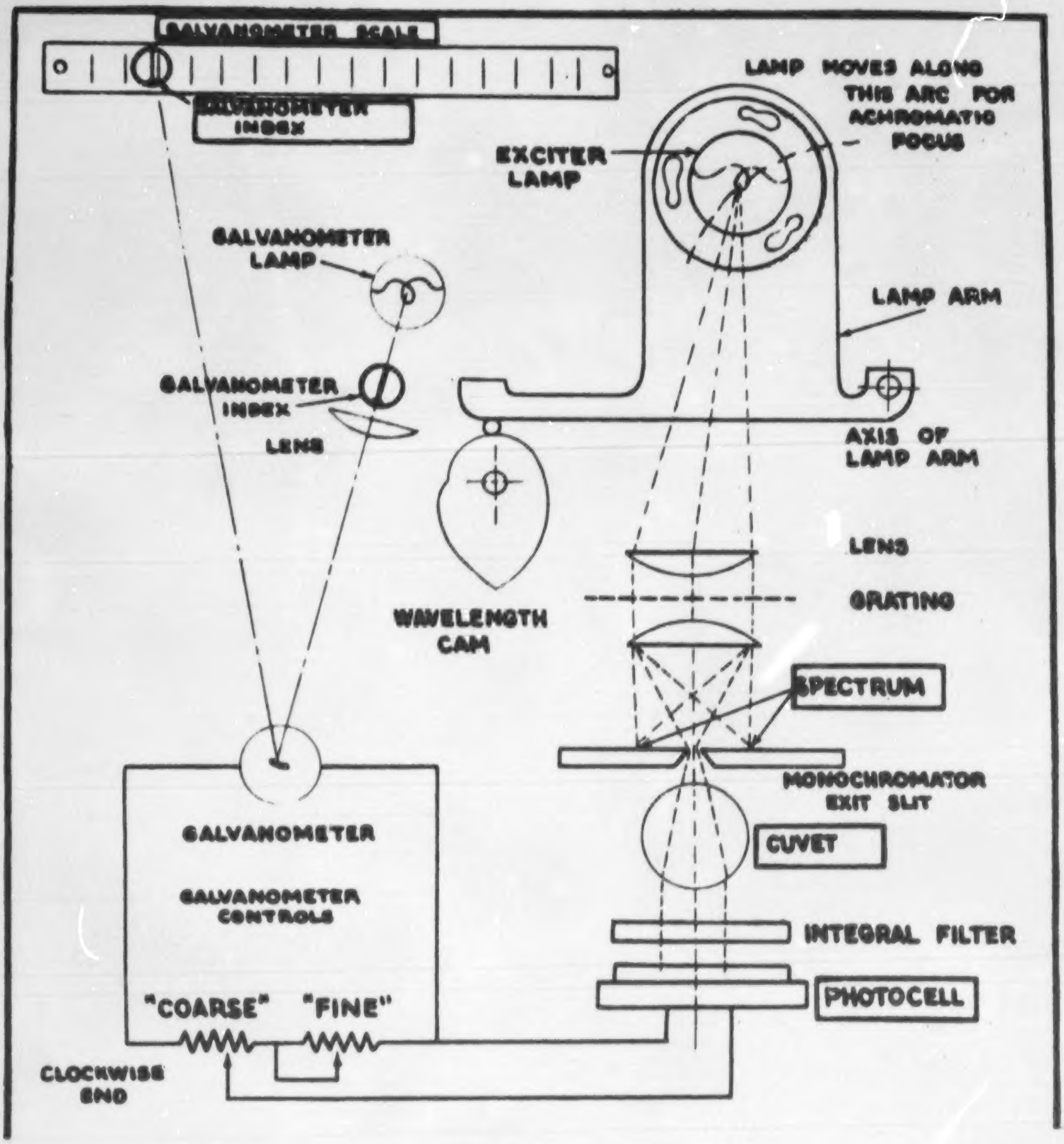

GALVANOMETER SCALE
GALVANOMETER INDEX
LAMP MOVES ALONG THIS ARC FOR ACHROMATIC FOCUS
EXCITER LAMP
GALVANOMETER LAMP
LAMP ARM
GALVANOMETER INDEX
LENS
AXIS OF LAMP ARM
LENS
GRATING
WAVELENGTH CAM
SPECTRUM
MONOCHROMATOR EXIT SLIT
CUVET
GALVANOMETER
GALVANOMETER CONTROLS
INTEGRAL FILTER
PHOTOCELL
"COARSE"
"FINE"
CLOCKWISE END

Figure 3. Schematic diagram of the Coleman Junior II and the Coleman Junior 6 A spectrophotometer optical systems.

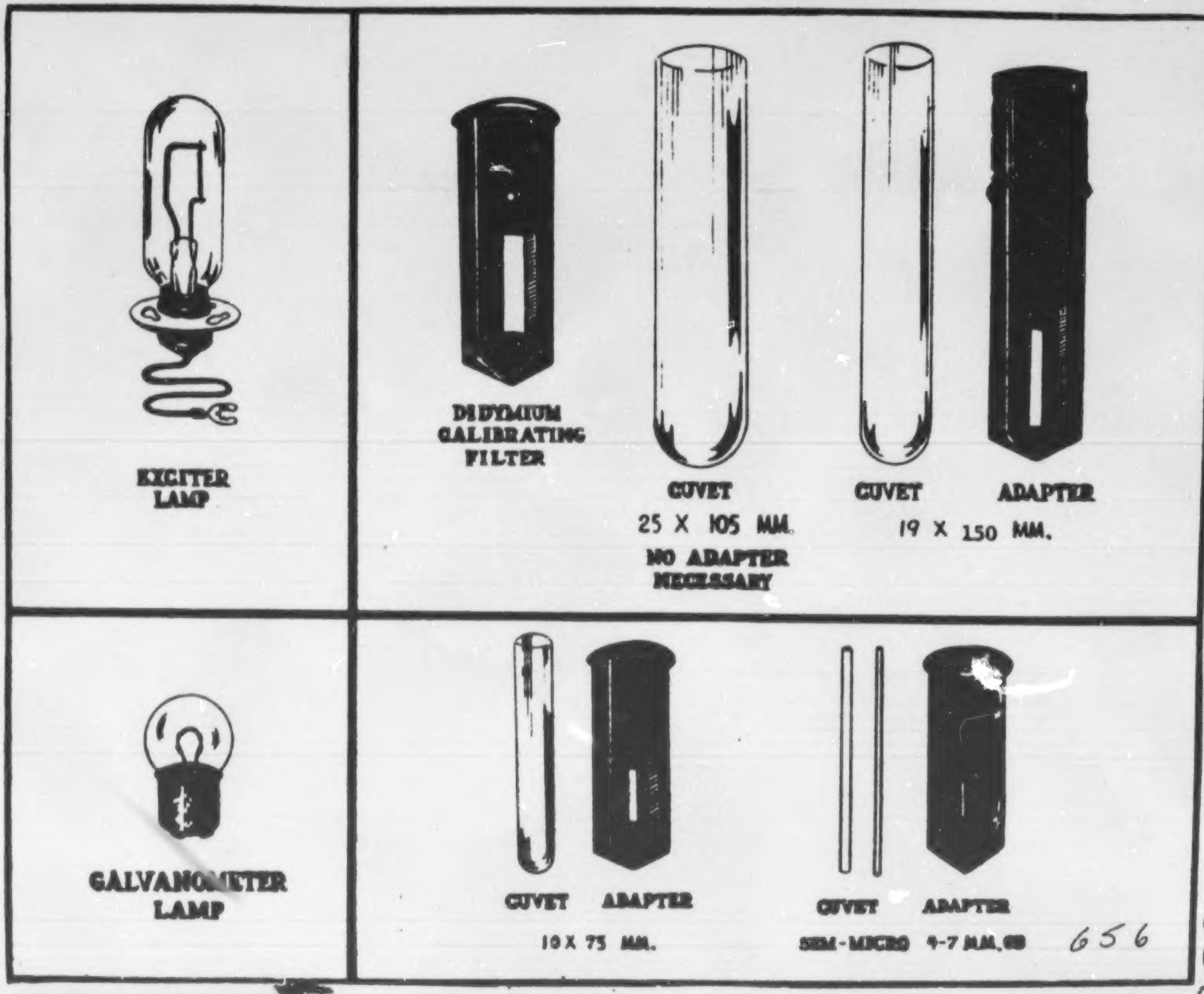
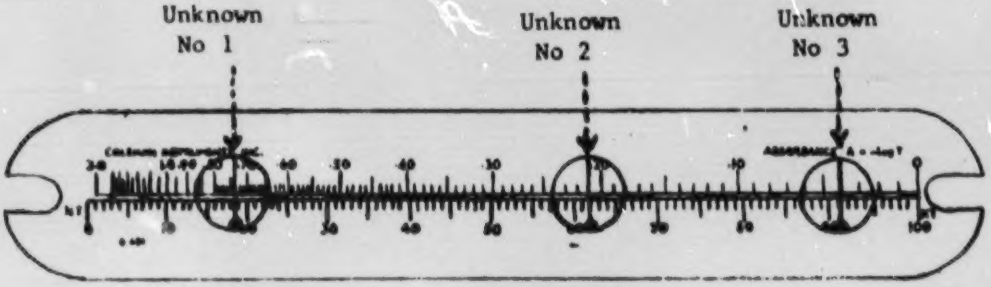

EXCITER LAMP
DIDYMIUM CALIBRATING FILTER
CUVET
25 X 105 MM.
NO ADAPTER NECESSARY
CUVET
ADAPTER
19 X 150 MM.
GALVANOMETER LAMP
CUVET
ADAPTER
10 X 75 MM.
CUVET
ADAPTER
SEMI-MICRO 4-7 MM. OD

Figure 4. lamps, cuvets and adapters for the Coleman spectrophotometers.

Example of Absorbancy Readings on Galvanometer Scale Panel



Sample No	Absorbancy
Unknown No 1	.74
Unknown No 2	.21
Unknown No 3	.04

In the above diagram, the bottom scale is the transmittance scale. It reads from left to right and is divided into equal divisions. It relates to the amount of light that passes through the cuvet. Accordingly, no light passage registers 0 percent T, and complete light passage registers 100 percent T on the galvanometer scale panel. Transmittance is normally read only for zeroing, blanking, and calibrating the spectrophotometer.

The top scale is the absorbance scale which reads from right to left. Complete light passage registers zero absorbance; no light passage registers greater than 2 absorbance (actually infinite absorbance). The divisions are not all the same size, and because of this, care must be taken when absorbance is read. A close inspection of the scale and the above examples should solve any problems in reading the absorbance scale. Absorbance is normally read for all standard, control, and unknown samples.

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COMMON SPECTROPHOTOMETRIC TECHNIQUES

Zeroing

After a spectrophotometer has had enough time to warm up, it must be zeroed. A cuvet adapter or a didymium filter is placed in the cuvet well and turned so that all light is blocked. The spectrophotometer is then set at zero percent T. If a slight adjustment is needed, the galvanometer scale panel is moved. If a greater adjustment is required, it is accomplished by moving the zero lever control.

Blanking

A spectrophotometer is used to measure the amount of light absorbed by a solution. In the clinical laboratory, it is generally used to measure the light absorbed after the combination of the substance being tested for and the proper reagents. These reagents, or even the sample, may absorb light; therefore, a blank is used to mechanically discount any absorbance due to the reagents or the sample. A blank may be just air (an air blank), all of the reagents with no sample added (a reagent blank), or the sample and all but the color producing reagent (a sample blank). After the spectrophotometer has been zeroed, the blank is inserted into the cuvet well. The machine is then set at 100 percent T by adjusting the 100 percent T course and fine controls. Every time there is a change in wavelength or a different test run, the spectrophotometer must be reblanked.

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073 641

CALIBRATION OF THE SPECTROPHOTOMETER

1. Warm up the spectrophotometer at least 15 minutes before use.

2. Set wavelength dial to read 610 nm. Look into the cuvet well to see if orange-red light is coming through the well, If not, call an instructor.

3. Zero the spectrophotometer. Block off the light and set zero percent T either by sliding the scale panel or by adjusting the zero lever control.

4. Blank the machine. An air blank is used with the didymium filter; therefore, with the cuvet well empty and the well covered with a cap, set 100 percent T with the coarse and fine controls.

5. Place the didymium filter in the cuvet well. Position it so that the light passes through the filter. Make sure that the key on the filter is fitted to the slot in the front of the cuvet well collar.

6. Read the percent transmittance (%T).

7. Each didymium filter has on it its correct percent transmittance (usually 45 or 46 percent T) at 610 nm. If a machine is correctly calibrated, it will read a percent transmittance within ± 1.5 percent of the value given on the outside of the filter. If an instrument is not calibrated correctly, call an instructor.

EXERCISE ON THE USE OF THE SPECTROPHOTOMETER

A. Procedure

1. Turn on the spectrophotometer; let it warm up for at least 15 minutes before use.
2. Obtain one tube of each unknown sample from the wire baskets supplied. Label each tube.
3. Transfer the contents of each tube into a correspondingly labeled large (19 X 150 mm) cuvet.
4. Zero the spectrophotometer.
5. Adjust the wavelength to 420 nm.
6. Blank the machine with a deionized water blank.
7. Read and record the absorbance of each unknown below.
8. Adjust the wavelength to 580 nm and repeat steps 6 and 7 above.

B. Questions

1. Why is there a change in absorbance when the wavelength is changed?
2. Why do these solutions have different absorbancies at the same wavelength of light?
3. If the above procedure had been run in small (10 X 75 mm) cuvets, what would have happened to the absorbance values? Why?

C. Results

SAMPLE	ABSORBANCE	
	420 nm	580 nm
Unknown 1	______	______
Unknown 2	______	______

D. Notes

1. Be sure to use only clean cuvets. Fingerprints, scratches, bubbles, and lint will interfere with the passage of the light.
2. Many test tubes resemble cuvets. Test tubes do not have the constant optical properties nor the constant internal diameter as cuvets. Only cuvets may be used in the spectrophotometer.

M 08-280-210-2
073 643

REFERENCE

Lee, L. W. Elementary Principles of Laboratory Instruments. 2d ed. St. Louis: C. V. Mosby Co., 1970, pp 55-58.

SP5 Alan B. Hatcher

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-210-3
PATHOLOGY AND LABORATORY SCIENCES DIVISION 123
CHEMISTRY BRANCH

Photometric Terms

RADIANT ENGERY. Energy transmitted as electromagnetic radiation.

WAVELENGTH. Distance, measured along the line of propagation, between two points in place on adjacent waves. (The peaks of waves are called maxima; valleys are called minima. The distance between two adjacent maxima or minima is a wavelength). The symbol for wavelength is λ, and the unit of measurement normally used in medical laboratories is the nanometer.

NANOMETER (nm). Unit of length equal to one-billionth of a meter (0.000000001 or 1×10^{-9} m). One nanometer is equal to 10 angstroms (Å).

ELECTROMAGNETIC SPECTRUM. An ordered arrangement of electromagnetic radiation, usually according to wavelength.

VISIBLE LIGHT SPECTRUM (daylight, white light). Radiant energy which is perceived by the normal eye. Approximate range--400 nm to 700 nm.

ULTRAVIOLET LIGHT SPECTRUM. The region of electromagnetic spectrum extending from 10 nm to 400 nm.

INFRARED LIGHT SPECTRUM. The region of electromagnetic spectrum from 700 nm to 300,000 nm.

SELECTIVE ABSORPTION. Ability of a substance to absorb certain wavelengths of radiant energy while transmitting others. The type of radiant energy which is absorbed is determined by the molecular or atomic structure of the substance.

PHOTOELECTRIC SPECTROPHOTOMETRY. The light absorptive capacity of a solution is measured by conversion of transmitted radiant energy into electrical current by a light sensitive element; the electrical current is in turn quantitated.

MONOCHROMATIC LIGHT. Light of one wavelength or color. This term generally means a narrow bank of light, but the dimensions of "narrow" band have not as yet been officially defined. (Bandwidth of Coleman Jr., Model II Spectrophotometer is about 20 nm; in Beckman DU Spectrophotometer it is about 1 nm, yet both beams are normally referred to a monochromatic light).

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*This mimeo supports LP 08-280-210, 014.

TRANSMITTANCE (T) AND PERCENT TRANSMITTANCE (%T). Expression of quantity of light which is transmitted through the solution; e.g., 0.5T (50 percent T) means that half of the light directed through the solution is transmitted.

ABSORBANCE (A). Same as the term optical density (O.D.). Expression of quantity of light which is absorbed by the solution.' Units of absorbance (A) are obtained from the algebraic expression of "Beer's Law" through a series of mathematical calculations until the working formula, $A = 2 - \log_{10}$ percent T, is obtained. This formula is used when conversion of percent transmittance (%T) into units of absorbance (A) is required; e.g., 38 percentT = A?, $2 - \log_{10} 38 = 2 - 1.58 = 0.42$ A.

BEER'S LAW. The absorbance of a substance is directly proportional to the concentration of the substance, times the length of the light path of the substance, times the molar absorptivity of the substance.

BLANK SOLUTION. A solution included in the determination to eliminate light loss due to reagents, "color" of specimen, and cuvets in order to obtain true A or percent T value. The true value is interpreted as the amount of light absorbed or transmitted due to tested substance only. The blank solutions fall into three main categories.

1. Reagent Blank--includes all the reagents used in the production of color from the unknown specimen, except that distilled water (or some other noncolor producing solution) is substituted for the specimen.

2. Specimen Blank--includes the specimen without color producing reagents.

3. Distilled Water Blank.

Occasionally a combination of the above mentioned blanks is desirable; e.g.., the instrument is set at 0 absorbance (A) with a water blank, the reagent blank is read and the A of the reagent blank subtracted from unknown and standard readings; this procedure serves as an additional measure to check the quality of reagents.

REFERENCE

Henry, R. J. Clinical Chemistry Principles and Technics. New York: Harper and Row, Publishers, 1964, pp 2-3.

SP/6 Robert A. Womack

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY M 08-280-210-5
LABORATORY SCIENCES DIVISION 086
CHEMISTRY BRANCH

Coleman Junior Spectrophotometers: Calibration Techniques

1. 610 Nanometer Calibration

Calibration of the spectrophotometer may be made by use of the didymium filter at 610 nm. Each didymium filter has on it its correct percent transmittance (usually 45-47 %T) at 610 nm. If the machine is correctly calibrated, it will read a percent transmittance ± 1.5 %T of the value given on the outside of the filter.

2. Minimum percent transmittance (min %T) wavelength calibration

This wavelength calibration procedure differs from that procedure using the didymium filter at 610 nm. Didymium filters fade with age so that their specified transmittance readings at 610 nm are no longer accurate. However, their minimum transmittance wavelength (absorbance maxima) does not change regardless of age and fading of the didymium glass. Therefore, old didymium filters, marked for use at 610 nm, may be used for this procedure (min %T at 585 nm) to satisfactorily calibrate the spectrophotometer.

a. Turn power switch on and allow a 15 minute warm-up period.

b. With spectrophotometers that have a filter selector, rotate filter selector knob to the VIS position.

c. Using the wavelength control knob, adjust the spectrophotometer to approximately 585 nm.

d. All work for calibration is done using the percent transmittance (%T) scale (the bottom, black scale).

e. Place the didymium filter (or min %T filter) into the cuvet well turned so that all light is blocked off to the photocell. The spectrophotometer is then set to read zero percent transmittance (0 %T). If a slight adjustment is needed, the galvanometer scale panel is moved; if a greater adjustment is required, use the zero lever control.

f. Turn the filter so that light passes through the filter to the photocell. Using the galvanometer COARSE and FINE control knobs, adjust the galvanometer readout to midrange (55 %T is convenient).

g. Observe the %T reading as the wavelength control knob is rotated to change the wavelength through the range 580 to 590 nm:

(1) If the minimum %T reading is at 585 ± 1 nm, the spectrophotometer can be considered calibrated.

(2) If the minimum %T reading is not at 585 ± 1 nm, notify an instructor immediately.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-215
LABORATORY SCIENCES DIVISION 018
CHEMISTRY BRANCH

Specimen Processing and Volumetric Glassware (2 hours)

COURSES PRESENTED TO: 92B20, Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES:

Tietz, N. E., ed. Fundamentals of Clinical Chemistry. Philadelphia: W. B. Saunders Company, 1970, pp 42-46, 7-16.

STUDY ASSIGNMENT: After class review notes, and M 08-280-215-1, -2, -5.

STUDENT UNIFORM AND EQUIPMENT: Laboratory notebook

TOOLS, EQUIPMENT, AND MATERIALS: M 08-280-215-1, M 08-280-215-2, M 08-280-215-5, issued at the beginning of the 1st hour.

PERSONNEL: One instructor (MOS 92B4H)

INSTRUCTIONAL AIDS: Chalk, Chalkboard, and erasers.

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture (1st hour), Practical exercise (2d hour).

I. **INTRODUCTION** (5 min)

A. Opening Statement: Before an accurate clinical chemistry result can be achieved, a proper specimen must be obtained from a patient who has been properly prepared. This responsibility falls on the medical laboratory technician. Even if it doesn't draw the blood, it is the laboratory that sets policy on specimen collection and insures that ward personnel drawing blood adhere to that policy. Once the specimen has been collected, accurate volumes must be delivered in order to perform clinical chemistry determinations correctly. A good basic knowledge of the laboratory glassware that you will be using daily in the laboratory is essential.

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*This lesson plan supersedes LP 08-280-215, 084

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B. Objectives

1. Select the most common use of volumetric flasks in clinical chemistry.

2. Select the two types of calibration used on laboratory glassware.

3. Select the types of pipets, the use, type of calibration, and the method of delivery for each.

4. With practice, correctly use volumetric glassware and equipment to prepare reagents and perform laboratory determinations.

5. Recognize the difference between serum and plasma.

6. Select the method(s) for processing blood to obtain serum or plasma.

C. Class Procedure and Lesson Tie-In - This class is a one hour lecture on specimen processing and the use of volumetirc glassware, followed by an hour of practical exercise using volumetric glassware.

II. EXPLANATION (35 min)

A. Specimens

1. Proper patient preparation

a. Random

b. Fasting

c. Postprandial

2. Collection

a. Clot-serum

b. Anticoagulants - whole blood, plasma

NOTE: In general, hemolysis should be avoided.

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3. Processing

    a. Centrifugation

        (1) Serum - from clot tube

            (a) Allow complete coagulation (20-30 min)

            (b) Separate clot from side of glass tube

            (c) Centrifuge (2000 RPM for 10 min)

        (2) Plasma - from tube containing anticoagulant

            (a) Centrifuge (2000 RPM for 10 min)

            (b) Separate supernatant fluid from cells

QUESTION: What are the three most common specimens collected for chemistry determinations?

ANSWER: Random, fasting, and post prandial

    b. Protein Free Filtrate

        (1) Precipitating agent or method

            (a) Essential that chemicals used do not interfere with clinical determination

            (b) pH of filtrate must be considered

        (2) Filtration or centrifugation

        (3) Incomplete protein precipitation - indicated by foaming

4. Preservation of Specimens

    a. Refrigeration

    b. Freezing

B. Volumetric Glassware

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1. Volumetric flasks

   a. Accuracy - the greater the capacity, the greater the accuracy

   b. Use

      (1) Preparation of solutions with concentration

         (a) Introduce proper amount of weighed solute

         (b) Fill approximately half full with solvent

         (c) Dissolve solute

         (d) QS to mark with solvent while mixing

         (e) Temperature effect

      (2) Reading the meniscus

2. Pipets

NOTE: Refer students to M 08-280-215-1

   a. Transfer (volumetric)

      (1) Accuracy - decreases with decrease in size

      (2) Common clinical use - standards and non viscous solution

   b. Oswald-Folin

   c. Micropipets

   d. Serological

   e. Eppendorf

   f. Repipets

NOTE: Refer students to M 08-280-215-2

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QUESTION: What type of delivery does the transfer pipet have?

ANSWER: Touch tip

3.' Pipetting technique

a. Propipet

b. Pipump

c. Pipetting procedure

4. Calibration

a. To contain

b. To deliver

NOTE: Refer Students to M 08-280-215-5

C. Questions From Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Specimens

2. Volumetric Glassware

B. Closing Statement: Accurate and precise clinical chemistry determinations are not only a service to the patient, but reflect professionalism on yourself and your laboratory. Without a good working knowledge of the equipment you use and the specimens required, you cannot achieve this goal.

IV. APPLICATION (45 min)

A. Instructor's Activities

1. Step-by-step, orient students to the use of the available equipment.

2. Supervise student's activities, making on the spot corrections of student technique.

B. Student Activities

1. Observe use of volumetric equipment.
2. Practice using volumetric equipment.
3. Clean up area and equipment as necessary.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY IM 08-280-215
LABORATORY SCIENCES DIVISION 018
CHEMISTRY BRANCH

Specimen Processing and Volumetric Glassware

Many steps are involved in achieving accurate and precise clinical chemistry determinations. The preservation of the chemical integrity of the specimen from the time of collection to the time of testing is of utmost importance if the results are to be meaningful. Not only must the laboratory equipment and glassware be prepared but often, the laboratory specialist must also prepare the patient prior to the collection of the blood.

In general, blood for chemical analysis should be drawn while the patient is in the postabsorptive state. An overnight fast is the usual procedure, although a six hour fast is often ample. During this time there is no need to restrict water intake. The common procedures that are affected most significantly by eating are glucose (elevated), inorganic phosphorous (decreased), thymol turbidity (increased), and triglycerides (increased). Random blood specimens can be collected for many tests and will not affect the results significantly. After a baseline from a fasting or random test is established, a postprandial specimen is often collected to indicate deficiencies such as low or inactive insulin levels in cases of diabetes.

In the collection of the specimen, utmost care should be taken to insure the specimen is not hemolized or that any intefering substances are not introduced into the specimen. Hemolysis may interfere with a number of chemical procedures and should be avoided. Several constituents, such as glutamic oxalacetic transaminase, lactic dehydrogenase, acid phosphatase, and potassium are present in large amounts in red blood cells, so hemolysis will significantly elevate the values for these substances.

If serum is to be used in the determination, a specimen is allowed to clot 15-20 minutes and then the serum should be separated from the red cells. Glycolysis can take place while the clot is allowed to retract, so the time allowed for clot retraction is dependant upon the procedure to be done.

If plasma is the specimen to be tested, then an anticoagulant is added to the blood and the plasma is removed from the cells. One important thing to consider in the selection of the anticoagulant is whether or not it will affect the chemical analysis. Anticoagulants can remove the constituents to be tested for or can be measured as part of the constituent and cause poor results.

Once the specimen has been collected and reaches the laboratory, it must be processed before any tests can be run. Processing usually entails logging the specimen in, noting the date and time of arrival to the laboratory and removing the serum or plasma from the red blood cells. After the blood has been allowed to clot and the tube has been reamed with a wooden applicator stick, it is centrifuged to pack the RBC's into the bottom of the tube and allow removal of the supernatant for testing. Once again, care should be used to avoid hemolyzing the specimen by reaming the clot excessively or overcentrifugation. At this time, the serum or plasma can be tested or stored for future testing. Storage can be at room temperature, refrigeration, freezing, or stored in the dark. The test to be performed on the specimen will dictate the type of storage for the specimen.

In chemistry, serum is preferred over plasma when testing constituents that are relatively evenly distributed between intracellular and extracellular compartments. The stability of the various constituents varies and should be researched and included in every lab's S O P.

Most clinical chemistry procedures require accurate measurements of volumes to be delivered. In precise work, it is never safe to assume that the volume delivered by any piece of equipment is exactly that amount indicated by the graduation mark. Ideally all volumetric apparatus should be either purchased with a calibration certificate or calibrated by the analyst.

Volumetric flasks should be thoroughly cleaned and dried with purified ethanol or acetone before calibration. The flask is weighed and then filled with CO_2 free H_2O. The meniscus is set at the graduation mark and then weighted again. Volumetric flasks are found in sizes ranging from 1-4000ml. The greater the capacity, the greater the accuracy. Volumetric flasks are used primarily in the preparation of solutions with known concentrations such as standards.

In general, there are two types of pipets used in clinical chemistry. These pipets are calibrated either TC or TD, meaning to contain or to deliver. The volumetric, or transfer, pipet is designed to deliver a fixed volume of liquid and consists of a cylindrical bulb joined at both ends to narrower glass tubing. A calibration mark is etched around the upper suction tube and the lower delivery tube is drawn out to a fine tip. Volumetric pipets are calibrated to deliver the volume specified. They are used for accurate measurements of aliquots of nonviscous samples, filtrates, and standard solutions. The reliability of the calibration of the volumetric pipet decreases with a decrease in size and therefore, special micropipets have been developed for chemical microanalysis.

Ostwald-Folin pipets are similar to volumetric pipets, but have their bulb closer to the delivery tip. They are used for measuring viscous fluids such as blood or serum. The Ostwald-Folin has an etched ring near the mouthpiece indicating it has a blow-out type delivery.

Another type of pipet is the graduated, or measuring pipet, also called a serological pipet. This is a plain, narrow tube drawn out to a tip and graduated uniformly along its length. These pipets are intende for delivery of predetermined volumes. The seroligical pipet also has a blow-out type delivery.

Since many of the solutions pipetted are toxic or caustic, pipetting devices were developed and are used to protect the laboratory technician. These devices fit over the mouthpiece of the pipets and draw the solution. The most common pipetting devices used are the pro-pipet, the pipump, and aspirator hoses.

The proper handling of the specimens and correct use of glassware is the first major step in achieving accurate and precise clinical chemistry determinations.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-215-1
LABORATORY SCIENCES DIVISION 103
CHEMISTRY BRANCH

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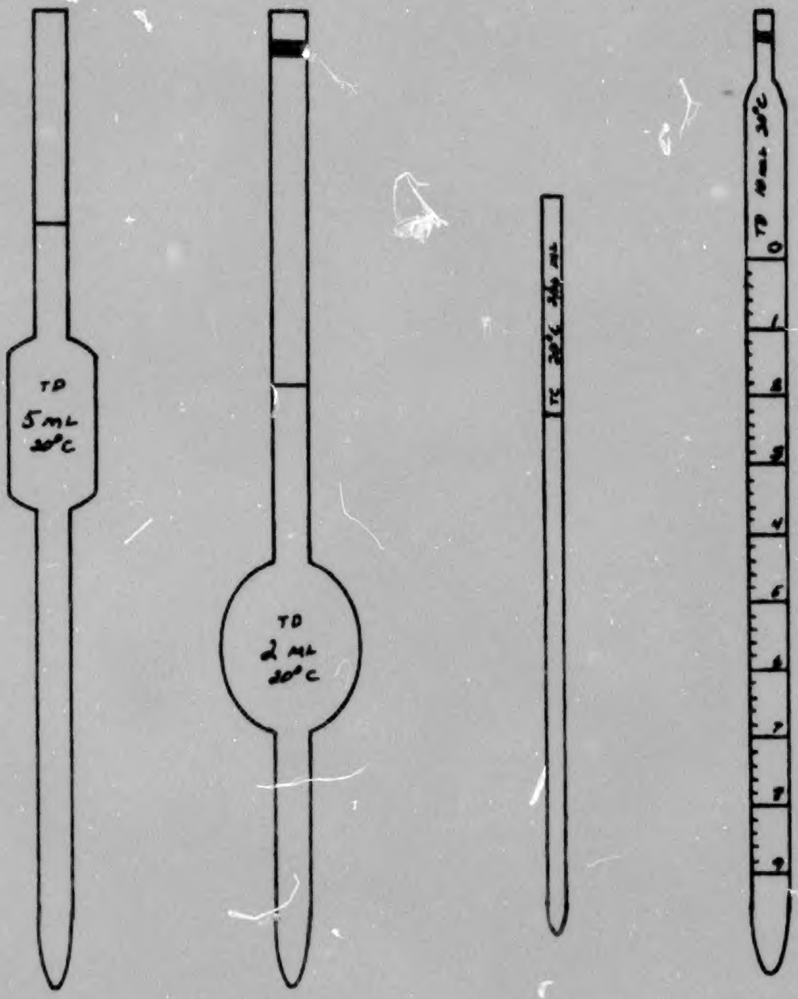
Classification of Pipets

Type of Pipet	Transfer (Volumetric)	Ostwald-Folin	Micropipets	Serological
Accuracy	Most accurate for aqueous solutions	Most accurate for viscous solutions	Most accurate for small quantities	Least accurate Advantages: a. Speed b. Delivery of intermediate volumes
Common Clinical Use	Aqueous Solutions (a) Dilutions of stock standards (b) Reconstitution of dehydrated controls. (c) Preparation and titration standard acids and bases.	Viscous Solutions (a) Blood (b) Plasma (c) Serum Aqueous Solutions (a) Some reagents (b) Some working standards	Small quantities of aqueous and viscous unknowns and standards	REAGENTS
Type of Delivery	To deliver (TD) Drain-out Touch delivery Do Not blow-out	To deliver (TD) Drain-out Blow-out "Frosted Band"	To contain (TC) Rinse-out (3-4 times)	Line to Line delivery Blow-out "Frosted Band"

*This mimeo supersedes M 08-280-340-3, 022.

Figure 1. Diagram of various types of pipets.


TD
5 mL
20°C
TD
2 mL
20°C
TC
20°C
TD
20°C
0
1
2
3
4
5
6
7
8
9

Type	TRANSFER	OSTWALD-FOLIN	MICRO	SEROLOGICAL
Available Sizes	100 ml	2 ml	0.02 ml	25 ml
	50 ml	1 ml	0.05 ml	10 ml
	25 ml		0.1 ml	5 ml
	10 ml		0.2 ml	1 ml
	5 ml			0.2 ml
	3 ml			0.1 ml
	1 ml			

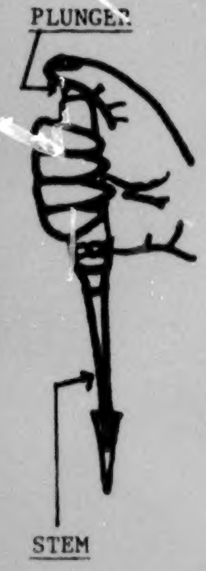
SP4 Richard L. Holmes 658

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-215-2
LABORATORY SCIENCES DIVISION 106
CHEMISTRY BRANCH

The Propipet and The Eppendorf Pipet

. The Eppendorf Pipet

The Eppendorf pipet is a manually operated, piston-activated pipet used for the pipetting of microliter (µl) quantities of fluids. (1 µl = 1 λ = .001ml) In use, a disposable plastic tip must be attached to the pipet, the sample being drawn into the tip only.



1. Fit a clean, dry tip onto the stem snugly enough for a good seal. NOTE: There are many different tip types available; use all of one type throughout any determination.
2. Press plunger to the first stop, where resistance is first encountered (colored ring is still visible).
3. Immerse end of tip in solution and allow plunger to slide back. Never allow the plunger to snap back.
4. Wipe off plastic tip being careful not to touch the end. NOTE: Never hold horizontal or lay pipet down while filled.
5. With tip slightly above center of surface of receptacle, dispense by pressing the plunger all the way down.
6. Touch off last drop on container wall.
7. Remove tip.

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*This mimeo supersedes M 08-280-215-2, 054.

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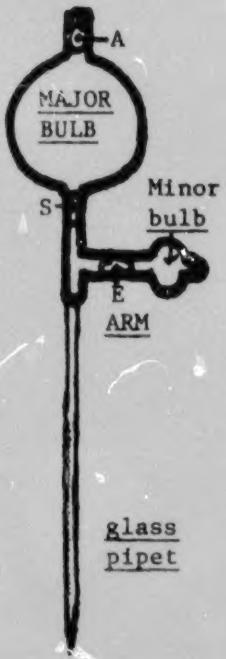
B. The Propipet



The Propipet is a rubber device of two bulbs and three valves used to assist in pipetting moderate quantities.

1. After inspecting pipet, moisten upper tip and insert into bottom of Propipet. NOTE: Keep hands close together to prevent breaking pipet and cutting hands.

2. Empty major bulb by squeezing A valve, pressing air out, and releasing A valve.

3. With tip in solution, aspirate liquid by squeezing S valve. Stop when meniscus is slightly above desired level.

4. After wiping off pipet, lower to exact level desired by squeezing E valve. NOTE: Never hold horizontal or lay pipet down while it is filled. Fluids must not enter the Propipet.

5. Dispense into receptacle by squeezing E valve and allowing to drain. For drain-out delivery, propipet may be removed; for blow-out delivery, cover hole in arm, squeeze E valve, and squeeze minor bulb.

6. Carefully remove the pipet from the Propipet.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
CHEMISTRY BRANCH

M 08-280-215-4
027

Quality Control

OBJECTIVES

1. Define quality control, standard, control, accuracy, precision, mean, standard deviation, coefficient of variation, random sampling, and Gaussian curve.

2. List and explain the four steps involved in establishment of quality control program.

3. Given a quality control graph, state which of the following abnormal conditions has occurred: out-of-control values, shift, or trend.

4. List possible sources of error in the performance of laboratory determinations.

5. With practice, plot control values on a quality control chart.

GLOSSARY OF QUALITY CONTROL TERMS

1. Quality control is a surveillance program that insures the reliability of laboratory test results.

2. Control is the same type of specimen that is to be tested. The concentrations of constituents in the control are known. The control is used as an internal check of the accuracy and precision of the test procedure.

3. Accuracy is the mesure of one's ability to attain a true value.

4. Precision is the measure of one's ability to reproduce results.

5. Mean is the arithmetic average.

6. Standard deviation is the range of dispersion (distribution) of values about their mean.

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*This mimeo supersedes M 08-280-215-4; 084

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M 08-280-215-4
027

7. Standard is usually a pure, aqueous solution of the constituent to be measured and has a known, accurate concentration. Its absorbancy is used for comparison with those of the unknowns in calculating their concentrations.

8. Coefficient of variation expresses tha standard deviation as a percentage of the mean.

9. Random sampling is the avoidance of setting fixed patterns of selection when setting up a test procedure.

10. Gaussian curve is a bell-shaped curve of normal distribution in which the mean is at or near the peak and equal numbers are distributed above and below the mean in a symetrical distribution pattern.

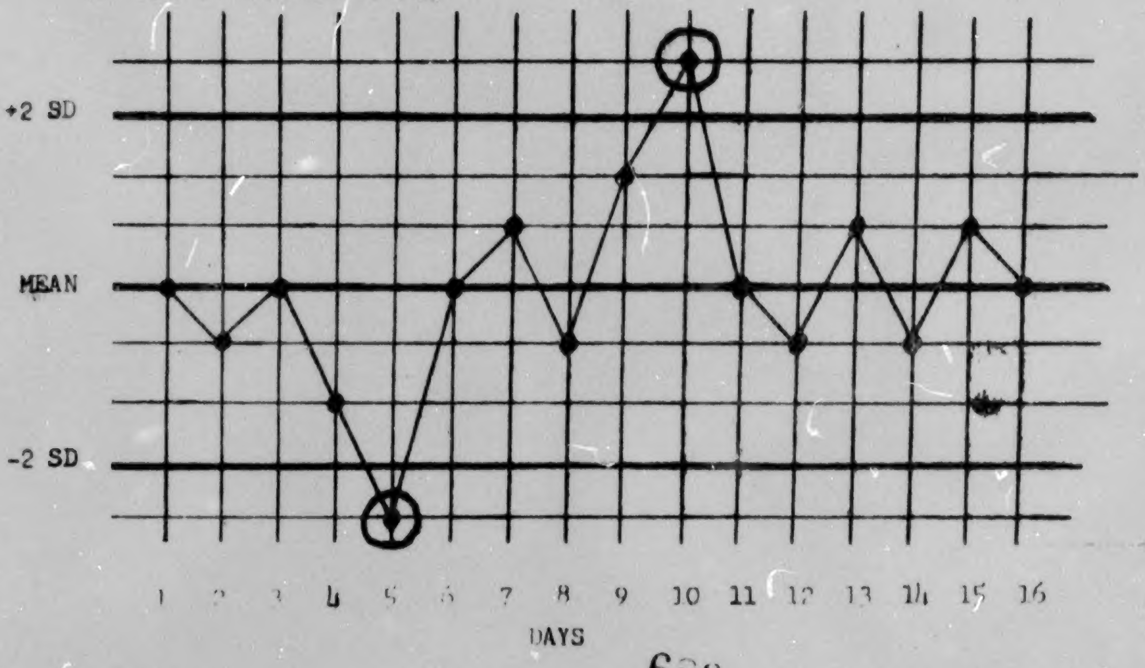
11. Out-of-control values are any values that have a difference from the mean greater than the limits of acceptable deviation.

12. Shift is six or more consecutive values to one side of the established mean.

13. Trend is six or more consecutively increasing or decreasing values.

GRAPHS

1. Out-of-control values



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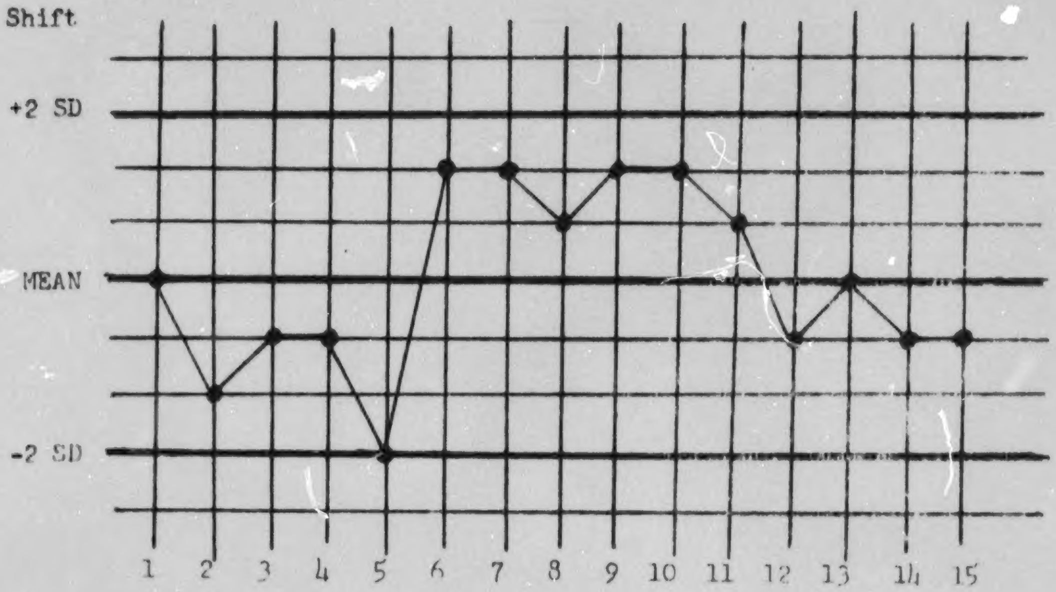
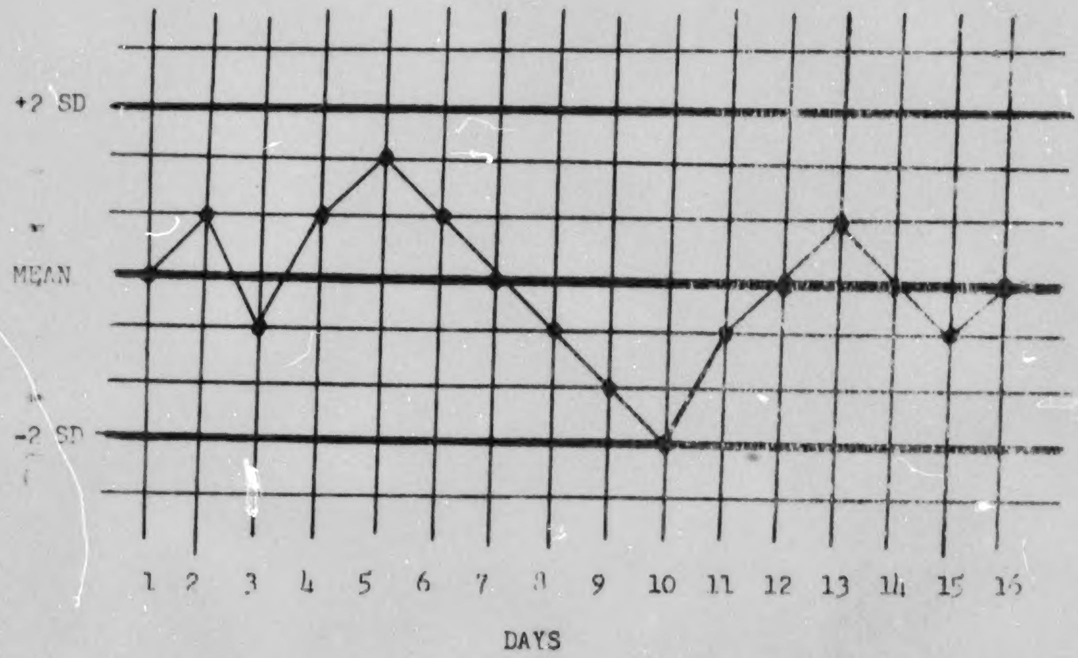
027

2. Shift



3. Trend



ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-215-5
LABORATORY SCIENCES DIVISION 113
CHEMISTRY BRANCH

Use of Pipets

1. In most cases, use the smallest pipet that will hold the desired volume.

2. Check the pipets for cleanliness and dryness.

3. Check the pipet tip for breaks.

4. Draw into the pipet the fluid to be measured, up to a point slightly above the desired graduation.

5. Wipe the outside of the pipet tip.

6. Maintaining the pipet in a vertical position, allow the meniscus level to come to the graduation line.

7. Carefully wipe the tip of the pipet but avoid absorbing any of the fluid from the inside of the pipet tip.

8. Discard the pipet-wipe immediately after using. The greatest source of cross-contamination can be the re-use of pipet-wipes.

9. Examine the pipet for air bubbles.

10. While delivering the fluid from the pipet, hold the tip of the pipet close to the level of fluid in the cuvet.

11. To read the meniscus in the pipet correctly, it may be necessary to tilt the cuvet slightly.

12. Touch the wall of the cuvet with the tip of the pipet to prevent a drop of fluid from remaining on the tip of the pipet.

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*This mimeo supports LP 08-280-215, 093.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-215-6
LABORATORY SCIENCES DIVISION 054
CHEMISTRY BRANCH

Sample Notebook Entry

While you are a student in the Clinical Chemistry Branch, you will be required to keep a daily record of the laboratory procedures that you perform. You will be graded for neatness and completeness of the notebook. The notebook should contain only the results from daily practical exercises and practical examinations. You should have the notebook in your possession at all times while in the laboratory-classroom. The following is an example of how a page of your notebook should be prepared.

TEST: Glucose
DATE: 7 Jan 74
WAVELENGTH: 630 nm
LIGHTPATH: 10 mm
SPECTROPHOTOMETER NUMBER: 253641

FORMULA

$$\frac{A_s}{C_s} = \frac{A_u}{C_u}$$

	ABSORBANCY	CONCENTRATION
STANDARD	0.50	200 mg/dl
CONTROL	0.25	100 mg/dl
UNKNOWN #1	0.22	88 mg/dl
UNKNOWN #2	0.35	140 mg/dl
UNKNOWN #3	0.20	80 mg/dl
UNKNOWN #4	0.40	160 mg/dl

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*This mimeo supersedes M 08-280-340-1, 022 and M 08-280-340-2, 022.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY LP 08-280-217
LABORATORY SCIENCES DIVISION 018
CHEMISTRY BRANCH

QUALITY CONTROL (1 hour)

COURSES PRESENTED TO: 311-92B10

PLACE: Classroom/Laboratory

REFERENCES:

Faulkner, W. R., King, J. W., and Damm, H. C., ed. Handbook of Clinical Laboratory Data. 2d ed. Cleveland: The Chemical Rubber Company, 1968, pp 111-132, 153-173.

Tietz, N. W., ed. Fundamentals of Clinical Chemistry. Philadelphia: W. B. Saunders Company, 1976, 2d Edition, Chap 2.

STUDY ASSIGNMENT: M 08-280-217-1, -2

STUDENT UNIFORM AND EQUIPMENT: Notebook

TOOLS, EQUIPMENT, AND MATERIALS: M 08-280-217-1, -2, issued one each per student at beginning of class period.

PERSONNEL: One instructor (MOS 92B4H

INSTRUCTIONAL AIDS: Chalk, chalkboard, eraser, and Quality Control Chart.

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

I. INTRODUCTION (5 min)

A. Opening Statement: The word "control" has become an important one in the vocabulary of laboratory technicians. Due to the speed that is required in most laboratory procedures it becomes necessary to use a system that enables us to observe the proficiency of our test procedures. The system devised for this purpose is termed "Quality Control".

B. Objectives

0606

1. Recognize the definitions of quality control, standard, control, reagent blank, accuracy, precision, and mean.

2. Given a quality control graph, select the acceptable limits of variation from the mean of control values, and recognize which of the following abnormal conditions has occurred: out of control, shift, and/or trend.

3. Select three possible sources of error and items to be monitored in the clinical laboratory.

4. With practice, plot control values on a quality control chart.

5. With practice, use controls in laboratory procedures for monitoring the accuracy of results.

C. Class Procedure and Lesson Tie-in

Quality control will be utilized throughout the course and is included on the final written examination.

II. EXPLANATION (35 min)

A. Definitions

1. Quality control - A system of monitoring the precision and accuracy of laboratory results.

2. Accuracy - the ability to attain the true value.

3. Precision - the ability to reproduce test results.

4. Reagent blank - a mixture of all reagents used in the test minus the specimen. The blank is used to set 100% transmittance, thereby discounting any absorbance due to reagents.

5. Standard - A pure aqueous solution of the constituent to be measured.

6. Control - A specimen of the same type as is being tested, which is used as an internal check of the accuracy and precision of the test.

7. Standard deviation - the amount of scatter (dispersion) about the mean.

8. Mean - the average of all the results for one specimen.

B. Sources of Error and Items Monitored by a Quality Control Program

1. Reagents, standards, and controls
2. Equipment
    a. Calibration logs
    b. Temperature logs
3. Technician
    a. Pipetting
    b. Procedure errors

QUESTION: How does a quality control program monitor the technician?

ANSWER: If the technician uses poor technique, his control will be out of range.

C. Establishment of Quality Control Program

1. Select surveillance items
    a. Controls
    b. Equipment
2. Collect data
3. Prepare graphs from data
4. Use graphs to monitor surveillance items and correct problems.

D. Evaluation of Control Graphs

1. Shift - six or more consecutive values that do not cross the mean.
    a. Change in reagents
    b. Change in controls and/or standard
    c. Change in equipment calibration

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2. Trend - six or more consecutively increasing or decreasing values that may or may not cross the mean.

   a. Gradual change in reagents

   b. Gradual change in controls and/or standard

   c. Gradual change in equipment calibration

QUESTION: Is it possible to have a trend that is also a shift?

ANSWER: Yes, as long as the increasing or decreasing values do not cross the mean.

3. Out of control - a value outside the acceptable limits of deviation from the mean.

   a. Acceptable limits are plus or minus 2 SD.

   b. Patients' results are not acceptable when control is out of range

   c. All blanks, standards, controls, and patients must be repeated if control is out of range.

   d. Out of control values must be posted on the graph.

   e. An acceptable control must be posted for the same day as the out of control day with reason (if possible) for the out of control value.

   f. If the out of control values continue, notify the supervisor.

E. Questions from Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Definitions

2. Items monitored by a quality control program

3. Establishment of a quality control program

4. Evaluation of control graphs

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B. Closing Statement: A quality control program is vital if a laboratory is to function efficiently. Efficiency is the name of the game when results that are reported affect the patients' well being. This makes it imperative that a quality control program be established and maintained to insure accurate laboratory results.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY IM 08-280-217
LABORATORY SCIENCES DIVISION 018
CHEMISTRY BRANCH

QUALITY CONTROL

The word "control" has become an important one in the vocabulary of laboratory technicians. Due to the speed that is required in most laboratory procedures it becomes necessary to use a system that enables us to observe the proficiency of our test procedures. The system devised for this purpose is termed "quality control".

Quality control is a system of monitoring the precision and accuracy of laboratory results. To some there may appear to be a very fine line between accuracy and precision. However, they are not synonymous and have two separate meanings. Accuracy is the ability to attain true values, whereas precision is the ability to reproduce test results. Therefore, it is quite possible to achieve precision while being totally inaccurate.

Quality control is set up to monitor the actual test procedures that are performed in the laboratory. In the chemistry section of the laboratory the procedures usually require the use of a reagent blank, standard, and control. The reagent blank is a mixture of all the reagents used in the procedure minus the specimen. The blank is used to set 100%T or 0 absorbance. This discounts any absorbance due to the reagents and allows only absorbance due to the specimen to be measured. A standard is a pure aqueous solution of the constituent to be measured that has a known concentration. With the understanding that the standard has a known concentration, it is possible to calculate unknown values based on the optical density of the known value and the optical density of the unknown. A control is a specimen of the same type as is being tested, which serves as an internal check of the accuracy and precision of the test parameters. The control also has a known value, and when calculated against the standard, the value of the control must fall within predetermined limits of its known value.

These predetermined limits that were mentioned are termed standard deviations. Standard deviation is a measure of the amount of dispersion of values about the mean, with the mean being the average of all the results for a given specimen such as a control. Acceptable values for a control must fall within plus or minus two standard deviations from the determined mean. This mean is established using the control values from the preceding month.

The quality control program monitors reagents, standards, controls, equipment, and the technician performing the procedures. If there is a problem with either reagents, standards, or controls, the value of the control, run in conjunction with the procedure, will not fall within the acceptable plus or minus two standard deviations from the established mean. likewise, if the technician uses poor technique while performing a procedure, it will be reflected by the failure of the control to fall within acceptable limits. The equipment used in the laboratory is also monitored in that calibration and temperature logs are kept daily.

In order to establish a quality control program surveillance items must be selected. These items include controls and equipment. Once the surveillance items are selected, data must be collected and graphs are prepared from this data. These graphs are used to evaluate the surveillance items and any problems that arise must be dealt with by the technician.

When evaluating these control graphs, the technician should be on the look-out for shifts, trends, and out of control values. A shift is comprised of six or more consecutive values that do not cross the mean. Shifts may occur if there is a change in reagents, controls or equipment calibration. A trend includes six or more increasing or decreasing values, that may or may not cross the mean. Therefore, it is possible to have a trend that is also a shift and vice-versa. Trends are usually due to a gradual change in reagents, controls, and equipment calibration. Out of control values are those values that fall outside of plus or minus two standard deviations from the mean. Out of control values may result from any inconsistency in the procedure, ranging from technician error to inadequate reagents. Anytime the control is out of range, the results of all other specimens measured in the procedure are considered invalid and the entire procedure must be repeated. All out of control values must be recorded on the control graph along with a repeated control that is within the established range. When the corrected value for the control is recorded, it should be accompanied by an explanation as to why the original value was out of control limits if possible. If, when repeated, the control values are still out of range, the supervisor should be notified immediately.

A quality control program is vital if a laboratory is to function efficiently. Efficiency is the name of the game when results that are reported affect the patients' well being. This makes it imperative that a quality control program be established and maintained to insure accurate laboratory results.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-220
LABORATORY SCIENCES DIVISION 084
CHEMISTRY BRANCH

Carbohydrates (9 hours)

COURSES PRESENTED TO: 311-92B20, Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES:

Arnow, L. E. Introduction to Physiological and Pathological Chemistry. 8th ed. St. Louis: C. V. Mosby Company, 1972, pp 242-249, 255-265.
Davidsohn, I. and Henry, J. B., ed. Todd-Sanford Clinical Diagnosis by Laboratory Methods. 14th ed. Philadelphia: W. B. Saunders Company, 1969, pp 549-559.
Tietz, N. W., ed. Fundamentals of Clinical Chemistry. Philadelphia: W. B. Saunders Company, 1970, pp 145-166.

STUDY ASSIGNMENT: After class, review M 08-280-220-1.

STUDENT UNIFORM AND EQUIPMENT: Slide rule and laboratory notebook.

TOOLS, EQUIPMENT, AND MATERIALS: M 08-280-220-1, issued one per student at the end of the second hour; M 08-280-220-2, issued one per student at the beginning of the first hour.

PERSONNEL: 1st, 2d, and 9th hours - one instructor (MOS 92B4H); 3d thru 8th hours - eight instructors (MOS 92B4H).

INSTRUCTIONAL AIDS: Chalk, chalkboard, eraser, and standard laboratory reagents and equipment for the O-toluidine glucose determination.

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: L (1st and 2d hr), PE (3d thru 8th hr), EX (9th hr)

FIRST AND SECOND HOURS

NOTE: Issue M 08-280-220-2, one per student. Students take lecture notes on this mimeo.

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*This lesson plan supersedes LP 08-280-275, 081.

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LP 08-280-220
084

I. INTRODUCTION (5 min)

A. Opening Statement: Carbohydrates are essential for body metabolism. The degradation of carbohydrates to carbon dioxide and water releases energy which is used by the body in its other metabolic processes. If there is a shortage of carbohydrates in the body or some inability to metabolize them, severe disorders can occur. Therefore, it is essential that you as medical laboratory specialists be able to perform determinations for glucose, one of the most important carbohydrates, both accurately and confidently.

B. Objectives

1. Briefly describe the composition and classification of carbohydrates.

2. Briefly outline carbohydrate metabolism in the body and list its influencing factors.

3. Outline the procedure of and state the need for a glucose tolerance test.

4. List the standard methods for the collection of laboratory specimens for glucose determinations.

5. State the principles of the three glucose methods.

6. State the normal values for serum and cerebrospinal fluid glucose concentrations.

7. With practice, perform glucose determinations.

C. Class Procedure and Lesson Tie-in

1. Two hours of lecture on carbohydrates to be followed by a six hour practical exercise and an hour's examination.

2. Glucose determinations will be performed throughout the chemistry block of instruction and as a major "STAT" procedure in all clinical chemistry laboratories

II. EXPLANATION (75 min)

A. Carbohydrates

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1. Composition

a. Elements

    (1) Carbon

    (2) Hydrogen

    (3) Oxygen

b. Ratio

    Two hydrogens to one oxygen

c. Radicals (functional groups)

    (1) Aldehyde

    (2) Ketone

2. Classification

a. Monosaccharides

    (1) One simple sugar unit

    (2) Examples

        (a) Glucose

        (b) Fructose

        (c) Galactose

b. Disaccharides

    (1) Two simple sugar units

    (2) Examples

        (a) Sucrose

            1 Glucose

            2 Fructose

        (b) Lactose

            1 Glucose

            2 Galactose

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(c) Maltose

1 Glucose

2 Glucose

QUESTION: Where in nature is maltose found?

ANSWER: In germinating grains and hops, two of the ingredients for making beer.

c. Polysaccharides

(1) Three or more simple sugar units

(2) Examples

(a) Starch

(b) Glycogen

NOTE: A starch or glycogen molecule may contain from 25 to 2500 glucose units.

QUESTION: What other polysaccharides can you name?

ANSWER: Cellulose, dextrins, pectins, and glycosides.

3. Metabolism

a. Utilization

(1) Glycolysis

$$\text{Glucose} \longrightarrow CO_2 + H_2O + \text{energy}$$

NOTE: This is a loose interpretation of the term glycolysis. Some authors limit glycolysis to only the anaerobic breakdown of glucose to form lactate.

(2) Amino acid production

b. Storage

(1) As glycogen

(a) Liver

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(b) Muscle

NOTE: Glycogen storage in the liver and muscles is limited to about one pound.

(2) As fat

NOTE: Glucose in excess of body needs for immediate metabolism and glycogen storage is converted to fat and stored as adipose tissue.

c. Hormonal control

(1) Insulin

(a) Production in the pancreas

QUESTION: Which cells of the pancreas produce insulin?

ANSWER: The beta chief cells in the Islets of Langerhans of the pancreas.

(b) Functions

1 Decreases blood glucose levels

2 Increases cell permeability to glucose

3 Polymerizes glucose to glycogen

QUESTION: If a patient has an overproduction of active insulin, what will happen to his blood glucose level?

ANSWER: The patient's blood glucose level will decrease (hypoglycemia).

(2) Other hormones

NOTE: See ANNEX A for an explanation of the roles of other hormones in carbohydrate metabolism

(a) Growth hormone

(b) Adrenocorticotrophic hormone (ACTH)

(c) Hydrocortisone

(d) Epinephrine

(e) Glucagon

(f) Thyroxine

B. Glucose

1. Patient conditions

a. Normoglycemia

(1) Serum - 70 to 110 mg/dl

(2) CSF - 40 to 70 mg/dl

b. Hypoglycemia

(1) Low blood glucose level

(2) High body insulin levels

NOTE: A marked decrease in blood glucose levels is often called insulin shock.

c. Hyperglycemia

(1) High blood glucose level

(2) Low or inactive body insulin levels

NOTE: Hyperglycemia is often the result of diabetes mellitus.

2. Specimen collection

a. Venipuncture

(1) Routine

(2) Larger volume of specimen

b. Capillary puncture

(1) Small volumes

(2) Pediatric patients

c. Glycolysis inhibition

(1) Fluoride (as NaF)

(a) Use with blood or CSF

(b) As an anticoagulant

- (2) Refrigeration
  - (a) 25°C up to 8 hours
  - (b) 4°C up to 72 hours

d. Clot tube

- (1) Allow to clot
- (2) Ream clot
- (3) Centrifuge
- (4) Separate serum from clot
- (5) Refrigerate serum prior to test

NOTE: Nonhemolyzed sera that have been separated from the clot can be used for glucose determinations up to 8 hours at room temperature and up to 72 hours if refrigerated.

3. Types of serum specimens

a. Fasting

- (1) Abstinence from food 8-12 hours
- (2) Most commonly used

b. Postprandial

- (1) Blood drawn after eating
- (2) Two hour postprandial (2 hr PP)

QUESTION: What is the serum specimen of choice for most chemistry determinations?

ANSWER: A fasting serum specimen.

C. Oral Glucose Tolerance Test (GTT)

1. Screening test

a. Fasting glucose

b. 2 hr PP glucose

NOTE: A 2 hr PP glucose value greater than 120 mg/dl is considered suspect of diabetes mellitus.

2. Patient's preparation
    a. Pre-test diet
    b. Fasting state
3. Test sequence
    a. Fasting specimens
        (1) Blood
        (2) Urine
    b. Glucose ingestion
        (1) Adults - 100 g glucose in 200 ml water
        (2) Children - 0.5 g glucose per pound body weight

NOTE: Caution should be exercised in administering the glucose dosage to a suspected severe diabetic. The dosage may be sufficient to cause diabetic acidosis. Recommend that the dosage be given under the direct supervision of the patient's physician.

    c. Timed specimens after glucose ingestion
        (1) Blood and urine
        (2) 1/2, 1, 2, and 3 hours
4. Glucose measurement
    a. Blood (serum) - quantitative
    b. Urine - qualitative
5. Test interpretation
    a. Renal threshold
        (1) Blood glucose level greater than 180 mg/dl

NOTE: The renal threshold varies among individual patients. A normal individual should not surpass the renal threshold.

(2) Excess glucose secreted in urine - glycosuria

b. Typical blood glucose changes

NOTE: See ANNEX B. Develop chart on the chalkboard as the explanation is presented.

(1) Normal curve

(a) Fasting - normal

(b) Peak level

1 30 to 60 minutes

2 Not over 170 mg/dl

(c) 2 hr level - less than 110 mg/dl

(2) Diabetic curves

(a) Mildly abnormal

1 Fasting - slightly elevated

2 Peak level - 30 to 60 minutes

3 2 hr level - still elevated (moderate)

(b) Severely abnormal

1 Fasting - moderately elevated

2 Peak level - about 2 hours

3 2 hr level - severely elevated, reaching peak, will remain elevated several more hours

(3) Hyperinsulinism curve

(a) Fasting - low normal or low

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(b) Peak level - 30 to 60 minutes, slight increase above fasting level

(c) 2 hr level - usually less than fasting level

6. Patient nausea

   a. Sweetness of glucose

   b. Stimulants

      (1) Smoking

      (2) Coffee

      (3) Tea

QUESTION: Which two body fluids are tested in a glucose tolerance test?

ANSWER: Blood (serum) and urine.

D. Laboratory Methods for Glucose

1. Glucose oxidase

NOTE: Place the reactions on the chalkboard. The students need not memorize the reactions.

   a. Hydrogen peroxide production

   $\text{Glucose} + H_2O \xrightarrow[\text{oxidase}]{\text{glucose}} \text{gluconic acid} + H_2O_2$

   b. Color development

   $\text{Chromogen} + H_2O_2 \xrightarrow{\text{peroxidase}} \text{colored compound} + H_2O$

   c. Specificity

      (1) Enzyme test

      (2) True glucose results

2. Alkaline ferricyanide

   a. Reduction of yellow ferricyanide

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b. Production of colorless ferrocyanide

$$\underset{\text{(yellow)}}{\underset{\text{Ferricyanide}}{Fe^{III}(CN)_6^{-3}}} \xrightarrow[\text{heat \& alkali}]{\text{glucose}} \underset{\text{(colorless)}}{\underset{\text{Ferrocyanide}}{Fe^{II}(CN)_6^{-4}}}$$

c. Measurement of color loss

(1) Beer's law not validated

(2) Calibration curve drawn

d. AutoAnalyzer method

e. Least accurate

Measures all reducing substances

3. Ortho-toluidine

a. Principle

$$\text{o-toluidine} + \text{glucose} \xrightarrow[\substack{\text{acetic acid} \\ \text{Thiourea}}]{\text{heat}} \text{green color}$$

b. Procedure

(1) Dispense o-toluidine reagent

(2) Add sample

(3) Boil mixture for color development

(4) Read absorbancies

(5) Calculate values

c. Precautions

(1) Boiling water

(2) Acetic acid

(3) Poisonous o-toluidine

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NOTE: Issue M 08-280-220-1, one per student.

QUESTION: Which of the three methods for glucose that we have just discussed yields the most accurate test results?

ANSWER: The glucose oxidase method, because it is an enzymatic method that is specific for glucose.

E. Questions from Students

III. SUMMARY (10 min)

A. Review of Main Points

1. Carbohydrates
2. Glucose
3. Oral glucose tolerance test (GTT)
4. Laboratory methods for glucose

B. Closing Statement: The glucose determination is one of the most important procedures performed in the clinical chemistry laboratory. It is often required as an emergency procedure in the treatment of diabetics. The physician depends to a great extent upon the results of the glucose determination to prescribe treatment for diabetic patients and to monitor the effects of such treatment. It is imperative that the medical laboratory specialist understand the basic principles of glucose determinations and be able to accurately and rapidly make these determinations.

THIRD THROUGH EIGHTH HOURS

IV. APPLICATION (260 min)

A. Students' Activities

1. Obtain reagents and specimens from the instructor
2. Perform the o-toluidine glucose determination
3. Record test results in laboratory notebooks
4. Calculate test values
5. Check coefficient of variation with instructor

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B. Instructors' Activities

  1. Prepare and issue reagents and specimens
  2. Supervise students' activities
  3. Make on-the-spot corrections of students' technique
  4. Check students' work for accuracy

V. CRITIQUE (10 min)

A. Questions from Students

B. Sources of Error

C. Safety and Procedural Precautions

D. Closing Statement: The importance of the glucose determination warrants extreme care and close observation of the technique employed. Practice and concentration are necessary for the optimum performance of this determination.

NINTH HOUR

VI. EXAMINATION (45 min)

A. Directions to Students

  1. Put away all lecture notes and texts
  2. M 08-280-205-1 and -3 may be used as references

B. Instructor's Activities

  1. Issue quiz sheet, one per student
  2. Supervise students' activities
  3. Clarify any vague points
  4. Collect and grade completed quiz sheets
  5. Reteach instructional material as necessary

C. Students' Activities

1. Place identificaiton on quiz sheet

2. Answer all questions as appropriate

3. Turn in completed quiz sheet to the instructor

D. Critique

ANNEX A
INSTRUCTIONAL AIDS

Roles of Hormones in Carbohydrate Metabolism

1. Growth Hormone and Adrenocorticotrophic Hormone (ACTH)

These two hormones are secreted by the anterior pituitary. Both have an antagonistic action to insulin and tend to raise blood glucose levels.

2. Hydrocortisone

Hydrocortisone and other 11-oxysteroids (11-Ketosteroids) secreted by the adrenal cortex stimulate gluconeogenesis. The effect of ACTH is probably mediated by its action on the adrenal cortex to stimulate the production of 11-oxysteroids. Since the latter tend to increase the blood glucose level and are antagonistic to insulin they are sometimes referred to as "diabetogenic" hormones. In individuals with Cushing's syndrome there is an overproduction of steroids owing to a tumor or hyperplasia of the adrenal cortex, and these individuals tend to show hyperglycemia. Conversely, individuals with Addison's disease have a primary adrenocortical insufficiency and show moderate hypoglycemia.

3. Epinephrine

This hormone, secreted by the adrenal medulla, stimulates glycogenolysis with resultant increase in blood glucose levels. Physical or emotional stress causes increased production of epinephrine and an immediate increase in production of blood glucose for energy requirements. Tumors of adrenal medullary tissue, known as pheochromocytomas, secrete excess epinephrine or norepinephrine and produce moderate hyperglycemia as long as glycogen stores are available in the liver.

4. Glucagon

Glucagon, which is secreted by the alpha cells of the pancreas, increases blood glucose levels by stimulating hepatic glycogenolysis. Glucagon has no effect on muscle glycogen.

5. Thyroxine

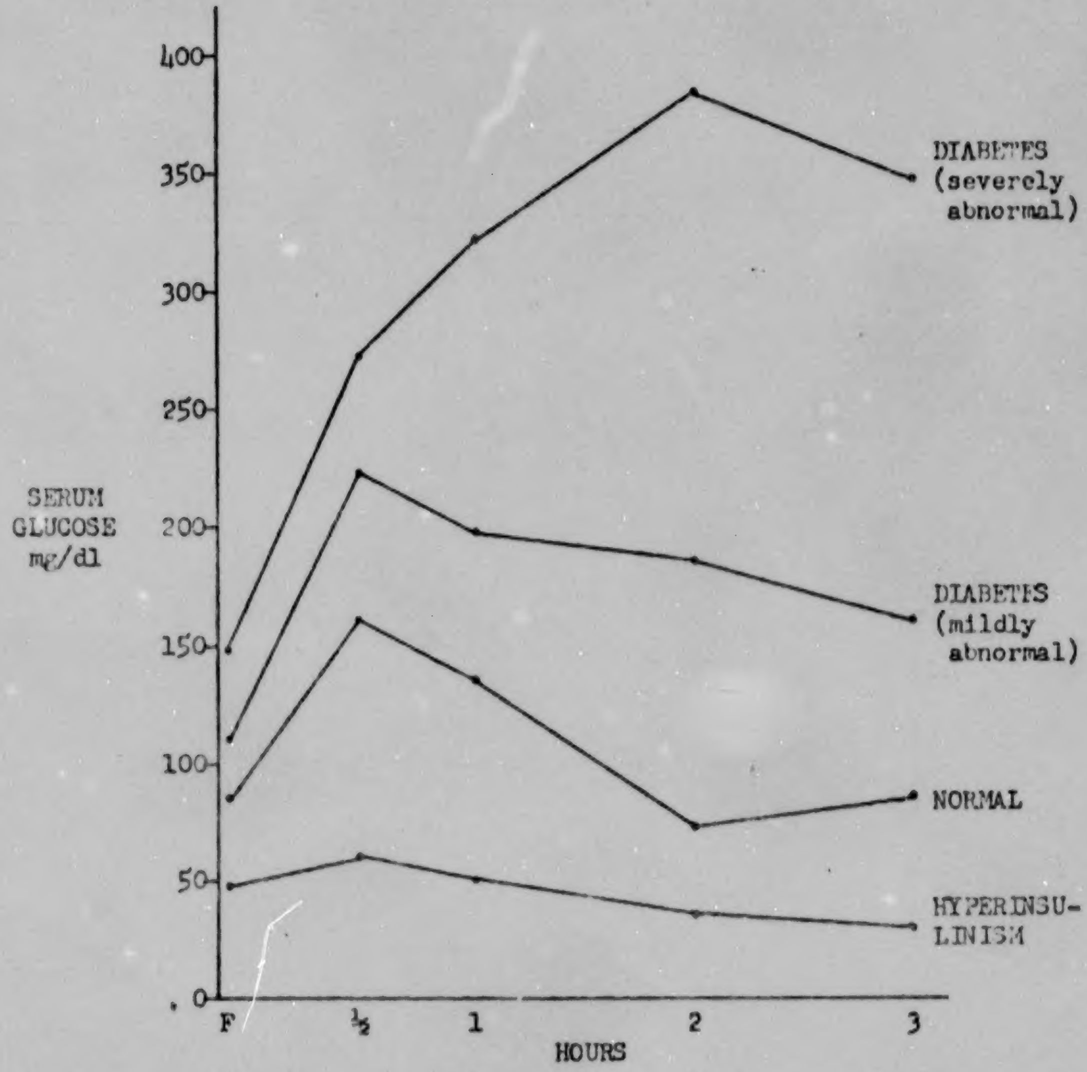
Thyroxine is secreted by the thyroid and appears to stimulate glycogenolysis. Individuals with thyroid conditions may show symptoms of mild diabetes and almost complete absence of liver glycogen. Thyroxine also increases the rate of absorption of glucose from the intestine.

NOTE: Used in part IIA3b(2) during the first hour.

ANNEX B
INSTRUCTIONAL AIDS

Chalkboard Drawing

Serum Glucose Changes for Oral 3 hr Glucose Tolerance Tests



NOTE: Used in part IIC5b during the second hour.

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ACADEMY OF HEALTH SCIENCES. UNITED STATES ARMY *IM 08-280-220
LABORATORY SCIENCES DIVISION 084
CHEMISTRY BRANCH

Carbohydrates

Carbohydrates are essential for body metabolism. The degradation of carbohydrates to carbon dioxide and water releases energy which is used by the body in its other metabolic processes. If there is a shortage of carbohydrates in the body, or some inability to metabolize them, severe disorders can occur. Therefore, it is essential that you as medical laboratory specialists be able to perform determinations for glucose, one of the most important carbohydrates, both accurately and confidently.

As their names indicate, carbohydrates are composed of atoms of carbon, hydrogen, and oxygen. Characteristically, the hydrogen and oxygen atoms are in the same ratio as in water - two hydrogens to one oxygen. The arrangement of the atoms in a carbohydrate molecule includes aldehyde and ketone radicals.

There are three basic classifications of carbohydrates; monosaccharides, disaccharides, and polysaccharides. The monosaccharides are composed of one simple sugar unit. Some examples of monosaccharides are glucose, fructose, and galactose. The disaccharides are a combination of two simple sugar units. Sucrose is a combination of glucose and fructose, lactose contains glucose and galactose; and maltose has two glucose units. Polysaccharides are a combination of three or more simple sugar units. Two of the more common polysaccharides are starch and glycogen, which may contain from twenty-five to twenty-five hundred (25 to 2500) glucose units.

Carbohydrates are used by the body for many important functions. One of the most important is the production of energy. Glycolysis is the process of breaking down glucose to produce energy. This is a series of chemical reactions with the net result being the oxidation of glucose to carbon dioxide, water, and energy. Many of the non-glucose carbohydrates ingested into the body are converted to glucose so that glycolysis can occur. Carbohydrates are used by the body for the production of other constituents besides energy. They also can be a metabolic source of amino acids and lipids. Glucose in excess of immediate body needs is stored as glycogen in the liver and muscle cells. The amount of glycogen that can be stored by the body is limited to about one pound. When the reserves are filled, the excess glucose is converted to fat and stored in the adipose tissue.

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*This instructor's manuscript supersedes IM 08-280-275, 081.

The body's use of glucose is regulated by many hormones; one of the most important is insulin, a pancreatic hormone. Insulin, produced by the beta chief cells of the Islets of Langerhans in the pancreas, causes a decrease in blood glucose level by promoting glycogenesis and lipogenesis (formation of fat from carbohydrates). Insulin also increases the cell's permeability to glucose and aids in polymerizing glucose to glycogen. Other hormones involved in carbohydrate metabolism are the growth hormone, adrenocorticotrophic hormone (ACTH), hydrocortisone, epinephrine, glucagon, and thyroxine.

A patient's condition, as far as his blood glucose level is concerned, is dependent upon insulin. An individual whose pancreas is producing large amounts of insulin will have a low blood glucose concentration, termed hypoglycomia. At the other extreme, an individual can be hyperglycemic if decreased or inactive insulin is produced. Hyperglycemia caused by an insulin deficiency is characteristic of diabetes mellitus. Normal individuals (normoglycemic) have serum glucose concentrations of seventy to one hundred ten (70-110) milligrams per deciliter and cerebrospinal fluid glucose concentrations of forty to seventy (40-70) milligrams per deciliter.

Blood specimens are collected for glucose and other clinical chemistry determinations primarily by venipuncture. In cases where only small amounts of specimen are readily available, such as pediatric patients, capillary puncture may be used. Steps must be taken to inhibit glycolysis by enzymes in the erythrocytes to prevent false, low serum glucose test values. Sodium fluoride is a glycolytic inhibitor that may be used with either blood or cerebrospinal fluid. Sodium fluoride is also an anticoagulant; if it is used, the glucose determination is performed on the resultant plasma. In a clinical chemistry laboratory, it is common for more than one test to be performed on a patient's blood sample. The use of anticoagulants may interfere with some of these tests. For example, the use of sodium fluoride, sodium heparin, or sodium citrate as an anticoagulant would completely invalidate a sodium determination. Because of these factors, most chemistry tests are performed on serum, but if a glucose determination is to be run, glycolysis must be inhibited. This may be accomplished by the quick processing of the clotted blood. After the blood specimen is drawn, it is permitted to clot for about 25 minutes. The clot is then reamed, the tube centrifuged, and the serum separated and refrigerated or frozen until the test is performed. The combination of rapid handling and refrigeration or freezing minimizes glucose loss through glycolysis. If these steps are not taken, the glucose concentration may be lowered as much as ten percent per hour due to the glycolysis by erythrocyte enzymes.

Generally, there are two types of sera used in a clinical chemistry laboratory. The most common is a fasting specimen; i.e., blood drawn from a patient who has had no food for eight to twelve hours. This is easily accomplished in a hospital by drawing the blood before the patient eats his breakfast. The other type of specimen used for clinical chemistry tests is the post-prandial specimen. Post-prandial literally means "after a meal"; blood is drawn at a set time after the patient has eaten. Since food has recently been ingested, the patient will be in a very active metabolic state. When the physician orders a two hour post-prandial (2 hr PP) specimen, he is ordering blood drawn two hours after the patient has finished eating.

A good diagnostic test for diabetes mellitus is the glucose tolerance test (GTT); i.e., how well will the patient react to an overloading dose of glucose. As a screening test, a comparison between fasting and two hour post-prandial blood glucoses is used. In a normal individual, the fasting glucose value should be normal and the two hour post-prandial glucose value should be less than one hundred ten (110) milligrams per deciliter. Individuals who do not follow this pattern are suspect as diabetics and should have a three hour oral glucose tolerance test performed.

Ideally, the patient should be placed on a high carbohydrate diet (1.75 grams of carbohydrate per kilogram of body weight) for the three days prior to the test to adjust his metabolism to the glucose overload administered in the test. The morning of the test, the patient reports to the laboratory in a fasting state; i.e., he has not eaten after the evening meal on the day before the test, although water may be taken.

Fasting blood and urine specimens are collected and the patient is given an oral dose of glucose, usually flavored with lemon juice or other sour fruit to cut the sweetness. Dosage is one hundred (100) grams for adults and one-half (1/2) gram per pound of body weight for children dissolved in about two hundred (200) milliliters of water. Blood and urine specimens are collected at one-half, one, two, and three (1/2, 1, 2, & 3) hours after ingestion of the glucose dosage. After each set of timed specimens is collected or, if glycolysis is inhibited after the entire test has been completed, the blood specimens are quantitatively measured and the urine specimens are qualitatively measured for glucose concentrations.

After ingestion of the glucose dosage, absorption is rapid and the blood glucose level rapidly rises. This stimulates the pancreas to produce more insulin, effecting a decrease of the blood glucose level as it is metabolized by the body. In a normal individual after ingestion of the glucose dosage, a peak blood glucose level is reached in thirty to sixty (30-60) minutes, usually less than one hundred seventy (170) milligrams per deciliter. The glucose is rapidly metabolized and the blood level at two hours after ingestion of the dose should be less than one hundred ten (110) milligrams per deciliter; often it is decreased to less than the fasting level because of overstimulation of the pancreas to produce insulin.

- Patients who have diabetes mellitus do not show this pattern because they have decreased production of, or inactive, insulin. The blood glucose rise is slower from an elevated fasting level, depending on the severity of the disease state. Blood glucose levels peak between thirty and sixty (30-60) minutes in mild cases and at about two hours in severe cases, usually higher than the renal threshold (about 180 mg/dl with variations among individuals and with age). Metabolism of the glucose is slow so that the two hour level remains elevated; moderately so in mild cases and just reaching the peak in severe cases, where elevated blood glucose levels may persist as long as three to four hours after ingestion of the dosage.

The glucose tolerance test may also be used in the diagnosis of other disease states. In patients with hyperinsulinism (chronic overproduction of insulin by the pancreas), the fasting blood glucose level is low or low normal. After ingestion of the glucose dosage, a peak blood level is reached in thirty to sixty (30-60) minutes; usually only a slight increase from the fasting level. Metabolism is rapid, with the two hour blood glucose level being lower than the fasting level.

During the glucose tolerance test, patient's nausea and/or vomiting may become a factor effecting the test. The large dose of glucose which is administered is sickly sweet unless it is flavored by the addition of juice from a sour fruit such as a lemon. Smoking or drinking stimulants such as coffee or tea may also cause the patient to feel nauseated. The patient should be instructed to avoid any of these factors. If the patient regurgitates all or part of the glucose dosage, the test is invalidated and the laboratory technician should be informed.

In clinical chemistry laboratories, several different methods can be used to quantitate body fluid glucose levels. The most accurate of the glucose methods is the glucose oxidase test. The enzyme, glucose oxidase, catalyzes the conversion of glucose to gluconic acid and hydrogen peroxide. In a secondary reaction, the hydrogen peroxide and an oxygen-accepting chromogen are converted by the catalytic effect of peroxidase to form a colored complex. The intensity of this color is measured spectrophotometrically and is proportional to the glucose concentration in the sample. This method yields "true" glucose values since the enzyme is specific for glucose. Substances in the specimen which will inhibit the peroxidase reaction are eliminated by the use of a Somogyi protein-free filtrate prior to the secondary reaction.

The alkaline ferricyanide glucose method makes use of the fact that glucose will reduce the yellow ferricyanide ion to produce the almost colorless ferrocyanide ion. Because this color fade is not proportional to the specimens' glucose concentrations, Beer's law is not valid and a

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calibration curve must be used to determine the glucose concentrations. This method is not as accurate for glucose as the glucose oxidase method since there are other reducing substances (such as glutathione and creatinine) in the specimen that will also react with the ferricyanide. Accuracy is increased with the use of dialysis in automated procedures and this method is widely used for glucose determinations on the Autoanalyzer.

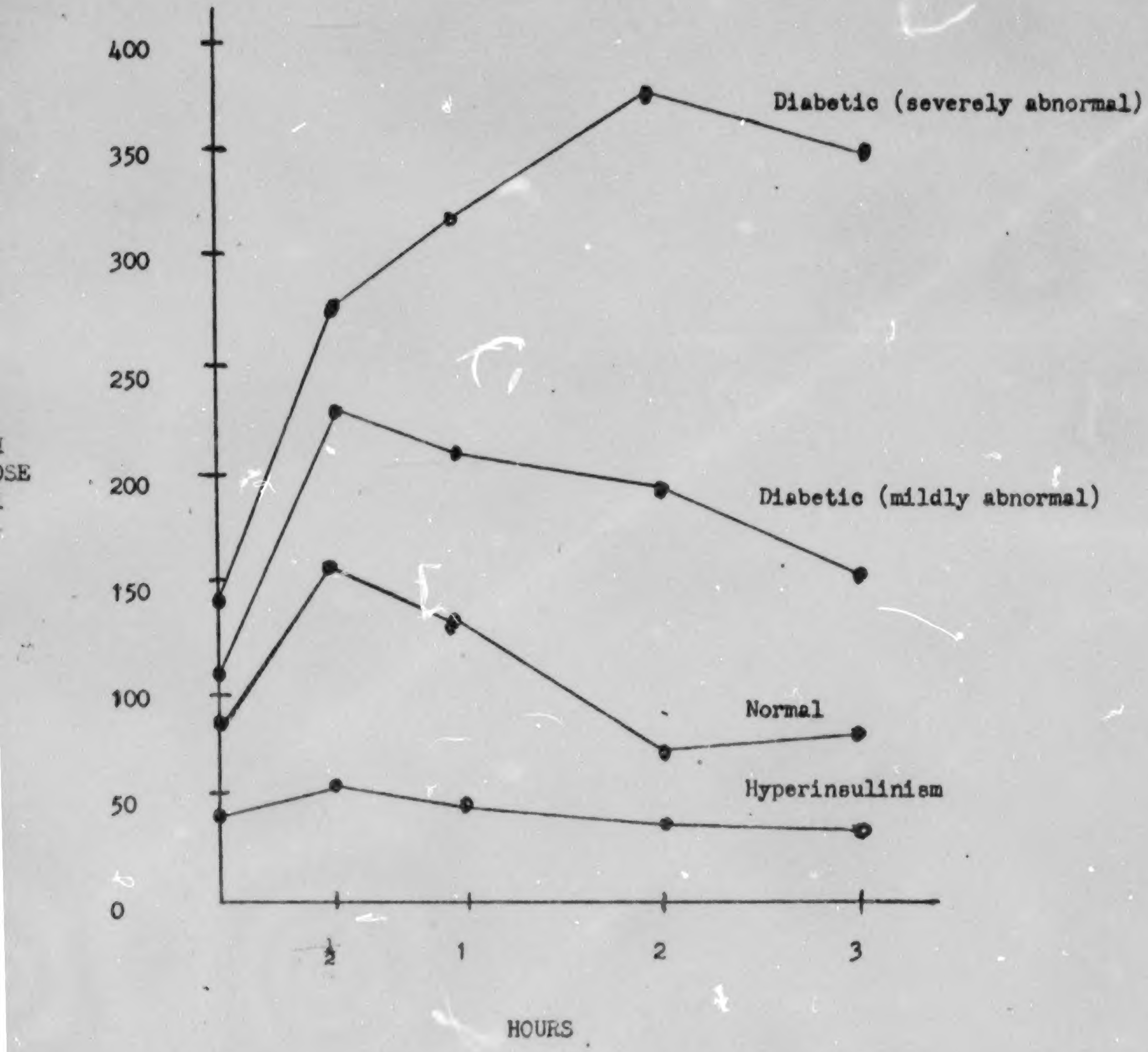
The ortho-toluidine method yields glucose values that closely agree with those obtained with the glucose oxidase method. The ortho-toluidine method will be used in our laboratory practical exercises. Glucose reacts with ortho-toluidine in hot glacial acetic acid to form a green complex. The intensity of the color follows Beer's law up to a serum glucose level of at least two thousand (2000) milligrams per deciliter. The intensity of the color is measured spectrophotometrically and is proportional to the specimen's glucose level. The ortho-toluidine reagent is poisonous and possibly carcinogenic, and contains glacial acetic acid. It should never be pipetted by mouth and excessive exposure to the noxious vapors should be avoided. Thiourea is added to the reagent as an antioxidant, without which the blanks are pink in color.

In the laboratory we will pipet one and one half (1.5) milliliters of the ortho-toluidine reagent and twenty microliters (20 ul) of specimen or standard solution into large test tubes. This mixture is placed in a boiling water bath for ten (10) minutes and then cooled to room temperature. The solutions are transferred to small cuvets and the absorbancies are read at 630 nanometers on the spectrophotometer, with a reagent blank of the ortho-toluidine reagent used to zero the instrument. The glucose concentrations of the specimens are calculated, using the modified Beer's law equation by ratio and proportion. Several precautions must be taken in this test procedure. Be careful not to be burned by the boiling water bath and remember that the ortho-toluidine reagent is poisonous and acidic.

During this period of instruction, we have discussed the composition and metabolism of carbohydrates, the clinical significance of blood glucose levels and their hormonal control, the glucose tolerance test, and several methods of quantitating body fluid glucose concentrations.

The glucose determination is one of the most important procedures performed in the clinical chemistry laboratory. Quite often, it is required as an emergency procedure in the treatment of diabetics. The physician depends to a great extent upon the results of the glucose determination to prescribe treatment for diabetic patients and to monitor the effects of such treatment. It is imperative that the medical laboratory specialist understand the basic principles of glucose determinations and be able to accurately and rapidly make these determinations.

Glucose Tolerance Test results charted on graph



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Interpretation of Glucose Tolerance Test results

Curve	Condition	Fasting	Peak Level	2 Hour Level
Normal	Normoglycemia Normal values: Serum: 70-110 mg/dl CSF: 40-70 mg/dl	Normal	30-60 Minutes Not over 170 mg/dl	less than 110 mg/dl
Hyperinsulinism	Hypoglycemia Caused by hyperinsulinism ↑insulin ↓glucose	Low normal or low	30-60 minutes Slight increase above fasting	Usually less than fasting level
Diabetic (mildly abnormal)	Hyperglycemia Caused by hypoinsulinism ↓insulin ↑glucose	Slightly elevated	30-60 minutes Increase above fasting	Still moderately elevated
Diabetic (severely abnormal)	Hyperglycemia May be caused by inactive body insulin level	Moderately elevated	Severely elevated, upon reaching peak will remain high for several hours	Elevated

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Sp4 Brian Estelle

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
CHEMISTRY BRANCH

*M 08-280-220-1
067

Body Fluid Glucose (GOD/PAP, Trinder Method)

A. PRINCIPLE:

Glucose is oxidized by the specific enzyme glucose oxidase (GOD) in aqueous solution to gluconic acid and hydrogen peroxide.

$$\text{Glucose} + O_2 + H_2O \xrightleftharpoons{\text{GOD}} \text{gluconic acid} + H_2O_2$$

The hydrogen peroxide reacts in the presence of a peroxidase (POD) with phenol and a chromogen (4-aminophenazone) forming a red dye product.

$$H_2O_2 + \text{phenol} + \text{4-aminophenazone} \xrightleftharpoons{\text{POD}} \text{red dye} + 2H_2O$$

The intensity of the color formed is proportional to the glucose concentration and can be measured spectrophotometrically at 500 nm.

B. REAGENTS:

1. Glucose reagents are obtained from B.M.C. kit #15715 "Auto-Test Blood Sugar." This kit should be stored at 4-8°C. Shelf life is approximately one year.

2. This B.M.C. kit contains the following components:

   a. Buffer/Enzymes (Reagent #1)

   This bottle contains: 100 mM phosphate buffer, pH 7.0
   >1.2 U/ml POD (25°C)
   >1.2 U/ml GOD (25°C)

   Non-reactive preservatives are added to assure stability

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* This mimeo supersedes M 08-280-220-1, 103

b. Phenol (Reagent #2)

This bottle contains 4.25 M phenol in 57% ethanol.

WARNING: Keep bottle closed and avoid exposure to direct light. For stability refer to expiration date on package label.

c. 4-Aminophenazone (Reagent #3)

This bottle contains 1.48 mM 4-aminophenazone.

3. Glucose working reagent:

   a. Add 3.5 liters of deionized water to a 4 liter Ehrlenmeyer flask.
   b. Add 10.0 ml of phenol from Reagent bottle #2. Use caution during this addition.
   c. Mix solution until phenol has dissolved (~30 sec.).
   d. Add the entire contents of one bottle of Reagent #1. Mix until dissolved (~5 min.).
   e. Add contents of one bottle of Reagent #3. Mix until dissolved (~5 min.).
   f. Add deionized water to the 4 liter mark and stir. Dispense into an amber glass container.

C. PROCEDURAL NOTES:

1. Working reagent must be stored in the refrigerator when not in use. When properly stored the reagent is stable and reactive for 4-6 weeks. Any indication of pink color, turbidity, or crystallization is cause for discarding the reagent.
2. Working reagent must be brought to room temperature before use in the procedure.
3. Working reagent is the same as the glucose reagent used for the Coulter Chemistry Unit (CCA-22). If need be, reagent can be used straight from the instrument without modification.

4. Interfering substances include mannose, altrose, and galactose which react at 1% of glucose. Maltose, ascorbic acid, glutathione, or uric acid do not react. Hemolyzed specimens should not be assayed as the hemoglobin color would be measured the same as the red dye produced from the chromogen thus yielding higher absorbancies.

5. Xanthrochromic or grossly bloody CSF samples require deproteinization prior to analysis.

6. At room temperature, red blood cells will utilize glucose at a rate of approximately 5% per hour. Unless sample was collected with a glycolytic inhibitor, such as sodium fluoride (NaF), the glucose determination must be run immediately.

7. This procedure may be run by incubating all tubes at 37°C for ten minutes or at room temperature for 30 minutes.

D. NORMAL VALUES:

1. Plasma or serum: 70-110 mg/dl
2. CSF: 40-70 mg/dl

E. CLINICAL SIGNIFICANCE:

1. Normal values - normoglycemia
2. High values - hyperglycemia (possibly diabetes mellitus)
3. Low values - hypoglycemia

F. PROCEDURE:

1. Add 2.0 ml of working glucose reagent to micro cuvets labeled for blank, standard, control, and unknowns.

2. Add 0.02 ml (20 lambda) of standard, control, and unknown solutions to their respectively labeled cuvets.

Summary of steps 1 and 2:

	Blank	Standard	Control	Unknowns
Working glucose rgt.	2.0 ml	2.0 ml	2.0 ml	2.0 ml
Standard (200 mg/dl)	----	0.02 ml	----	----
Control	----	----	0.02 ml	----
Unknowns	----	----	----	0.02 ml

3. Mix all tubes by vortex.
4. Incubate all tubes at room temperature for 30 minutes.
5. Read and record the cuvets absorbance at 500 nm within 30 minutes after completion of the incubation period. Use the blank to set 100% T on the spectrophotometer.
6. Calculate the results using Beer's Law.

G. CALCULATIONS:

1. Beer's Law is obeyed up to 400 mg/dl. Patient specimens which give apparent values above this point should be diluted (1:3) with saline and reassayed.
2. Formula:

$$K \text{ factor} = \frac{C_s}{A_s}$$

$$K \times A_u = C_u$$

C_s = concentration of standard

A_s = absorbance of standard

A_u = absorbance of unknown

C_u = concentration of unknown

3. Example calculations:

$$\frac{C_s}{A_s} \times A_u = C_u \text{ in mg/dl of glucose}$$

If: Concentration of standard (C_s) = 200 mg/dl
Absorbance of standard (A_s) = 0.450
Absorbance of unknown (A_u) = 0.146

Then:

$$K = \frac{C_s}{A_s} = \frac{200 \text{ mg/dl}}{0.450} = 444.4$$

$$K \times A_u = C_u \text{ in mg/dl}$$

$$444.4 \times 0.146 = 65 \text{ mg/dl}$$

4. Answers should be reported to nearest whole number in mg/dl.

H. REFERENCES:

1. Trinder, P. Ann. Clin., 6,24 (1969)

2. Henry, R.J. Clinical Chemistry, Principles and Technics, 2nd ed. Harper and Rowe Publishers, Inc. (1974) p. 1288.

3. Tietz, N.W. Fundamentals of Clinical Chemistry, W.B. Saunders Co. Philadelphia. (1976)

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-225
LABORATORY SCIENCES DIVISION 018
CHEMISTRY BRANCH

Protein and Kidney Function (11 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic)

PLACE: Classroom/Laboratory

REFERENCES:

Davidsohn, I., and Henry, B. H. Todd-Sanford Clinical Diagnosis. 15th ed. Philadelphia: W. B. Saunders Company, 1974, pp 561-569.

Ganong, W. F. Review of Medical Physiology. 7th ed. Los Altos Calif: Lange Medical Publications, 1975 pp 213-216, 526-527, 211-229, 351, 513-521.

Lehninger, A. L. Biochemistry. 6th ed. New York: Worth Publishers, 1970, chap 3-8.

STUDY ASSIGNMENT: After Class, study lecture notes and review M 08-280-225-2.

STUDENT UNIFORM AND EQUIPMENT: Laboratory notebook

TOOLS, EQUIPMENT, AND MATERIALS: M 08-280-225-2 issued one each per student at the beginning of class.

PERSONNEL: One instructor (MOS 92B4H) first and second hours.
Eight instructors (MOS 92B4H) third through eleventh hours.

INSTRUCTIONAL AIDS: Chalkboard, chalk, eraser, overh[illegible] projector, screen, and T 08-280-225-1-5.

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture (1st and 2d hours), Practical exercise (3d through 11th hours).

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*This lesson plan supersedes LP 08-280-225, 084.

LP 08-280-225
018

I. INTRODUCTION: (5 min)

A. Opening Statement: Ten thousand human beings die every week because of a severe deficiency in their diet, a deficiency in protein. Their deaths are not quick or simple but slow, agonizing torture. Young children suffer the most; their stomachs enlarge up to ten times the normal size with the arms and legs becoming nothing but bone covered with skin. Proteins are essential for life and without a minimum intake of them a person has no chance for survival.

B. Objectives

1. Select the four chemical elements found in all proteins.
2. Select the function of the peptide bond in protein molecules.
3. Recognize the four major functions of proteins in the body.
4. Select the two major classifications of proteins and the components of each.
5. Recognize the toxic and relatively non-toxic end-products of protein breakdown, and the respective organs responsible for the creation and removal of urea.
6. Select the two major functions of the kidney.
7. Select the three structural units of the nephron and the major function of each unit.
8. Recognize the range of normal values for serum total proteins and blood urea nitrogen.
9. Recognize the clinical significance of elevated or decreased blood urea nitrogen values.
10. With practice, satisfactorily perform blood urea nitrogen determinations.

C. Class Procedure and Lesson Tie-In

1. Two hours of lecture out of a total of eleven hours on proteins and kidney function.
2. Lecture will be followed by nine hours of practical exercise in the determination of blood urea nitrogen.

II. EXPLANATION (80 min)

A. Elemental and Structural Composition

1. Elemental composition
    - a. Common elements found in all proteins
        - (1) C - carbon
        - (2) H - hydrogen
        - (3) N - nitrogen
        - (4) O - oxygen
    - b. Other elements found in some proteins
        - (1) S - sulfur
        - (2) Mg - magnesium
        - (3) Fe - iron

NOTE: Show T-08-280-225-1 (See Annex A) to illustrate the following point.

2. Structural units-amino acids
    - a. Subunits of proteins
    - b. Organic acids with amine (NH_2) group attached
    - c. Formed by specific arrangement of C,H,O, & N
    - d. Twenty common amino acids found in body proteins
3. Structural composition of proteins
    - a. Type, number, and arrangement of amino acids determine specific chemical nature of proteins

NOTE: Show T-08-280-225-2 (See Annex A) to develop following point

- b. Amino acids are linked sequentially to form protein chains by formation of peptide bonds

NOTE: The peptide bond acts as the sole covalent linkage between amino acids in the backbone structure of proteins. Proteins usually contain more than one amino acid chain

c. Molecular weight of proteins ranges from approx. 4,000 to over 1,000,000 amu

NOTE: An amino acid chain must achieve a molecular weight of 4,000 before it can be considered a protein. Chains with weights below 4,000 are called peptides (made of few amino acids) or polypeptides (made of many amino acids). This range of 4,000 to over 1,000,000 is purely arbitrary.

QUESTION: What four basic elements are found in all proteins ?

ANSWER: Carbon, oxygen, hydrogen, and nitrogen

B. Classification of Proteins
(Two major classifications based on composition)

1. Simple proteins

   a. Protein composed only of amino acids

   b. Protein chain(s) contain no other major organic or inorganic groups

   c. Examples

      (1) Insulin

      (2) Albumin

      (3) Globulin

2. Conjugated proteins

   a. Protein composed of amino acids and a non-protein portion called a prosthetic group

NOTE: A prosthetic group may be organic or inorganic in nature and is covalently bound to the amino acid chain(s) of the protein

   b. Examples

      (1) Hemoglobin-prosthetic group is iron protoporphyrin

(2) Lipoprotein - prosthetic groups include cholesterol, phopholipids, and neutral fats

(3) Enzymes - prosthetic groups include a wide range of covalently bound organic molecules called coenzymes

C. Major Functions of Proteins

1. Structural

a. Make up structural components of cells

b. Examples

(1) Lipoproteins - in cell membranes

(2) Collagen - in tendons

(3) Myosin - in skeletal muscle

2. Maintainence

a. Maintain internal environment (homéostasis)

b. Examples

(1) Immunoglobulins - act as antibodies

(2) Plasma proteins - aid in coagulation

3. Regulation

a. Control of various metabolic pathways

b. [illegible]amples

(1) Hormones - regulate overall pathways

(2) Enzymes - regulate individual reactions within the pathways

4. Secondary energy source

a. Indirect energy source when glucose levels are low

b. Indirect energy source when amino acid levels are high

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QUESTION: Upon breakdown a protein is found to be composed of only amino acids. How would you classify this protein?

ANSWER: It would be classified as a simple protein.

D. Protein Metabolism

1. Ingestion

Proteins are the main nitrogen source for body

NOTE: Nitrogen is a basic element in the structure of the genetic material, (DNA) and in various high energy compounds such as adenosine triphosphate (ATP)

2. Digestion

a. Body cannot use animal or vegetable proteins in the forms found in food sources

b. Ingested proteins broken down by hydrolysis - the addition of water across peptide bond

(1) Into polypeptides in stomach by action of pepsin (an enzyme)

(2) Into individual amino acids in small intestine by action of various enzymes

NOTE: The proteolytic enzymes in the small intestine are pepsin, trypsin, chymotrypsin, carboxypeptidase, and leucine aminopeptidase.

QUESTION: What are the four main functions of proteins in the body?

ANSWER: Structure, maintainence, regulation, secondary energy

3. Absorption

Amino acids absorbed into blood stream through the wall of the small intestine

4. Utilization

a. By all body tissues to make structural proteins and some enzymes

b. By liver tissue

   1. To make specialized enzymes

   2. To make plasma proteins (Normal value -- 6-8 g/dl)

   3. Interconversion to other amino acids

c. By glandular tissue to make hormones

d. By RES system to make antibodies

5. Catabolism of excess amino acids

NOTE: Show T-08-280-225-3 (See Annex A) to illustrate the following points

a. Transamination

   (1) Transfer and collection of amine groups

   (2) Occurs to majority of amino acids (rest have individual pathways)

   (3) Amino groups from various amino acids transferred to a specific carrier molecule; forms one specific amino acid

NOTE: The carrier molecule is alpha-ketoglutarate; the amino acid formed is primarily glutamic acid. Alanine and aspartic acid continue through transamination pathways until they become glutamic acid.

   (4) Alleviates need for different pathway for each amino acid

b. Oxidative deamination

   (1) Occurs in the liver

   (2) Specific amino acid formed, broken down enzymatically into ammonia (NH_3) and an alpha-keto acid

      (a) Alpha-keto acid can be used to make glucose (gluconeogenesis)

      (b) Ammonia toxic and must be removed

c. Conversion of ammonia to urea

(1) Occurs in the liver (ornithine cycle)

(2) $CO_2 + 2NH_3 \dashrightarrow$ Urea (enzymatic reaction)

(3) Urea relatively non-toxic

(4) Removed by kidneys

E. Kidneys

1. Major functions

a. Excretion

(1) Removal of waste products

(2) Waste products formed during metabolism

(a) Urea and nitrogenous by-products

(b) Urobilin - waste product of hemoglobin breakdown

b. Regulation

(1) Control overall concentration of blood's constituent molecules by removing excess water or excess solute molecules

(2) Example - control of pH (acid-base balance) by removing bicarbonate molecules

2. Gross structure

a. Paired organs

b. Located in small of back

NOTE: Show T-08-280-225-4 (See Annex A) to illustrate the following points

3. Microanatomy - nephron

a. Functional unit of kidney

b. Approximately 1 million per kidney

c. Three basic structural components

  (1) Glomerulus

    (a) Dense capillary bed

    (b) Extraction of blood flowing into renal vessels

    (c) Filters all solutes from blood except high molecular weight proteins and cells

  (2) Bowman's capsule

    (a) Surrounds glomerulus

    (b) Collects filtrate

  (3) Tubular reabsorption system

    (a) Composed of convoluted tubules and Loop of Henle

    (b) Reabsorption of non-waste products from the filtrate

    (c) Reabsorption takes place along entire length of tubules

    (d) Substances reabsorbed include water, glucose, and amino acids

    (e) Waste products enter collecting tubules and travel to bladder via ureters

4. Kidney function tests

  a. Urinalysis - physical and chemical analysis of the urine

  b. Serum analysis

    (1) Creatinine

    (2) Urea nitrogen

      (a) Major end-product of protein metabolism

      (b) Constitutes 50% of urinary solids

F. Blood Urea Nitrogen (BUN)

1. Clinical significance

a. Normal values: 5 - 25 mg/dl

b. Values elevated in

(1) Dehydration or any disease resulting in excess water loss

(2) Excessive protein destruction

(3) Heart failure

(4) Most profoundly elevated in renal disease

c. Decreased values are of little clinical significance

2. Laboratory methods

a. Urease method

(1) Indirect method

(2) Measures ammonia

b. Diacetyl monoxime-thiosemicarbazide method

(1) Direct method

(2) Thiosemicarbazide acts as a catalyst

NOTE: Show T 08-280-225-5 (See Annex A) to illustrate reaction

(3) Manual or automated method

III. SUMMARY (5 min)

A. Review of Main Points

1. Elemental and structural composition

2. Classification of proteins

3. Major functions of proteins

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4. Protein metabolism

5. Kidneys

6. Blood Urea Nitrogen

B. Closing Statement: Proteins are the framework around which our structural and metabolic processes are built. There are many complex reactions involving proteins and the kidney. Therefore, it is to our utmost advantage to understand the functions of these complex molecules in relation to that intricate organ called the kidney.

THIRD THROUGH ELEVENTH HOURS

IV. APPLICATION (395 min)

A. Instructors' Activities

1. Step-by-step, orient the students to the DAM-TSC BUN test procedure.

2. Provide specimens and reagents.

3. Supervise students' activities.

4. Direct students in correction of out-of-control test values.

5. Collect and grade students' reports.

B. Students' Activities

1. Review the DAM-TSC BUN test procedure.

2. Satisfactorily perform the DAM-TSC BUN test procedure.

3. Notify instructors of out-of-control test values and obtain directions for their correction.

4. Maintain laboratory notebook and report sheet.

5. Report test results to the instructors.

6. Clean up equipment and area as required.

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V. REVIEW (10 min)

A. Questions From Students

B. Review of Main Points

1. Technique

2. Calculations and records

3. Precautions

a. Technique

b. Safety

C. Closing Statement: With practice and careful technique, the BUN test procedure may be performed with a high degree of accuracy and precision. This is important, not only because of the frequency of requests for the BUN values in the clinical laboratory, but because the same techniques are employed in many other test procedures.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *IM 08-280-225
LABORATORY SCIENCES DIVISION .018
CHEMISTRY BRANCH

Protein and Kidney Function

Ten thousand human beings die every week because of a severe deficiency in their diet, a deficiency in protein. Their deaths are not quick or simple but slow, agonizing torture. Young children suffer the most; their stomachs enlarge up to ten times the normal size with the arms and legs becoming nothing but bone covered with skin. Proteins are essential for life and without a minimum intake of them a person has no chance for survival.

All proteins are composed of four basic elements. These elements are carbon, hydrogen, oxygen, and nitrogen. Other elements may be found in proteins, including sulfur (which forms sulfhydryl bridges between protein chains), magnesium, and iron. These basic elements are bonded together to form amino acids, the sub-units of proteins. Amino acids are organic acids with an amine (NH_2) group attached to the alpha carbon. There are twenty common amino acids found in human proteins. These twenty amino acids differ from one another in only one small portion of their molecular structure. This variable portion, called an R group may be anything from a single hydrogen atom to a complex ring structure.

In the formation of a protein chain the type, number and arrangement of amino acids determines the specific chemical nature of the protein formed. How are specific proteins formed from these amino acids subunits? Proteins formation is accomplished by the sequential linkage of amino acids through the formation of peptide bonds. The peptide bond acts as the sole covalent linkage between amino acids in the backbone structure of proteins. The formation of the peptide bond is enzyme dependent. Complete functional proteins are usually composed of more than one amino acid chain. These chains are bound together through action of sulfhydryl bond formation between cysteine residues within the chains.

Proteins are generally very large molecules. Their molecular weights range from 4,000 to over 1,000,000 amu. An amino acid chain must achieve a molecular weight of 4,000 before it can be considered a protein. Chains with weights below 4,000 are called peptides (made of few amino acids) or polypeptides (made of many amino acids). This range of 4,000 to over 1,000,000 is purely arbitrary.

Proteins vary widely in structure and in function; this makes classification difficult. There are, however, two major classifications based

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*This instructor's manuscript supersedes IM 08-280-225, 084.

on composition. Simple proteins are composed solely of amino acids; the protein chains contain no other major organic or inorganic groups. Examples of simple proteins include insulin, albumin, and globulin. The second classification is that of conjugated proteins. These are composed of amino acids plus a non-protein portion called a prosthetic group. A prosthetic group may be organic or inorganic in nature and is covalently bound to the amino acid chain(s) of the protein. Examples of conjugated proteins include hemoglobin, which has iron protoporphyrin as a prosthetic group; lipoproteins, whose prosthetic group may be cholesterol, phospholipids, or neutral fats; and enzymes whose prosthetic groups include a wide range of covalently bound organic molecules called coenzymes.

Proteins perform four major functions in the body. The first is a structural function, making up the structural components of cells. Examples of structural proteins include lipoproteins in cell membranes, collagen in tendons, and myosin in skeletal muscle. Proteins are also involved in maintaining the body's internal environment (homeostasis). Examples of proteins that perform maintainence functions include immunoglobulins which act as antibodies and plasma proteins that aid in coagulation. Proteins may also perform a regulatory function, controlling various metabolic pathways. Hormones regulate overall pathways while enzymes regulate individual reactions within pathways. Proteins can possibly act as a secondary energy source. They supply energy indirectly when glucose levels are low or when amino acid levels are high.

Protein metabolism is carried out by a wide range of complex metabolic pathways. However, we can describe this metabolism by a series of general steps. This series begins with the ingestion of the protein in food sources. Proteins are the main nitrogen source for the body. Nitrogen is a basic element in the structure of the genetic material (DNA) and in various high energy compounds such as adenosine triphosphate (ATP). After ingestion the proteins enter the stomach where the process of digestion begins. This process is carried out because the body cannot use animal or vegetable proteins in their ingested forms. Proteins are broken down by hydrolysis the addition of water across the peptide bonds. Digestion is a two step process. Initial breakdown into polypeptides occurs in the stomach by the action of the enzyme pepsin. Complete breakdown into individual amino acids occurs in the small intestine. This complete breakdown is carried out by a wide variety of proteolytic enzymes, including trypsin, pepsin, chymotrypsin, carboxypeptidase, and leucine aminopeptidase. After complete breakdown into amino acids absorption into the bloodstream across the wall of the small intestine takes place. Utilization by the body's cells is the next step in the overall metabolic process.

Amino acids are used by all body tissues to make structural proteins and some enzymes. The liver uses amino acids also to make specialized enzymes, plasma proteins, and new amino acids through an interconversion pathway. The normal values for plasma proteins produced by the liver are 6-8 g/dl. Glandular tissue requires amino acids in the synthesis of hormones, as does the RES system to make antibodies.

If the body has a large excess of amino acids, or has no immediate need for them, it begins catabolic pathways to break down these amino acids. The first step in catabolism is a transamination pathway. Transamination is the transfer and collection of amine groups. This process occurs to the majority of amino acids; the rest go through their own individual pathways. During transamination the amino groups from various amino acids are transferred to a specific carrier molecule to form one specific amino acid. The carrier molecule is an alpha-ketoglutarate, and the amino acid formed is primarily glutamic acid. Alanine and aspartic acid continue through transamination pathways until they become glutamic acid. The reasoning behind transamination is that it condenses the breakdown into one specific pathway, alleviating the need for a different pathway for each amino acid.

Transamination is followed by oxidative deamination in the liver. The specific amino acid formed, glutamic acid, is broken down enzymatically into ammonia (NH_3) and a corresponding alpha-ketoacid. The alpha-keto acid can be used to make glucose (gluconeogenesis) or new amino acids. Ammonia is highly toxic to the body and must be removed.

Ammonia is converted to urea in the liver through a series of enzymatic reactions (ornithine cycle). Urea is relatively non-toxic and is removed from the body by the kidneys. In removing urea from the body the kidneys are performing one of their two major functions that of excretion.

Physiologically, excretion is the removal of waste products from the body. Waste products are formed during metabolism and include urea and nitrogenous by-products, and urobilin, a waste product of hemoglobin breakdown. The second major function performed by the kidneys is regulation. By regulation we mean the control of the overall concentration of the blood's constituent molecules by removing excess water or excess solute molecules. An example of this is the control of acid-base balance (pH) by removing bicarbonate molecules.

An understanding of the gross and microanatomy of the kidneys will help us to understand how these organs carry out their major functions. The kidneys are paired organs located in the small of the back. The nephron is the functional unit of the kidney. There are approximately one million nephrons per kidney, and each has three basic structural components.

Blood flowing through the renal arteries first encounters the glomerulus. The glomerulus is a dense capillary bed and blood flowing through it is under high pressure. This enables the filtration of all solutes from the blood except high molecular weight proteins and cells. Bowman's capsule surrounds the glomerulus and collects the filtrate. The filtrate, once collected, enters the tubular reabsorption system. This system is composed of the convoluted tubules and the Loop of Henle. The primary function of the tubular reabsorption system is the reabsorption of non-waste products from the filtrate. Reabsorption takes place along the entire length of the tubules. Substances reabsorbed include water, glucose, and amino acids. Waste products are not reabsorbed; instead, they enter the collecting tubules and travel to the bladder via the ureters for eventual removal from the body.

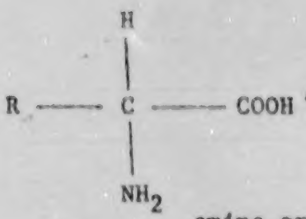
There are a number of ways the laboratory specialist can test to see if the kidney is functioning properly. One way is urinalysis, performing physical and chemical analysis on urine. A second method is serum analysis. We can test for levels of various waste products in the serum such as creatinine (the end-product of creatine metabolism) or urea nitrogen. Urea nitrogen is the major end-product of protein metabolism and constitutes 50% of urinary solutes.

The normal values for blood urea nitrogen (BUN) in serum range from 5 to 25 mg/dl. Values are elevated in dehydration or any disease resulting in water loss, excessive protein destruction, or heart disease, but are most profoundly elevated in renal disease. Decreased values are of little clinical significance. Laboratory methods for quantitation of BUN are varied. One method, by using the enzyme urease, measures ammonia formed during the test reaction. It is an indirect method. The diacetyl monoxime-thiosemicarbazide method is a direct method in which thiosemicarbazide acts as a catalyst. This method can be used either manually or as an automated procedure. In essence, the reaction involves reacting a color reagent containing diacetyl monoxime and thiosemicarbazide in the presence of an acid at 95-100° C for ten minutes with a known amount of serum. Urea nitrogen in the serum will react with the constituents in the color reagent forming a pink complex. This pink color can be measured spectrophotometrically at 520nm. Quantitation of urea nitrogen can be accomplished using Beer's Law.

Proteins are the framework around which our structural and metabolic processes are built. There are many complex reactions concerning proteins and the kidney. Therefore, it is to our utmost advantage to understand the functions of these complex molecules in relation to that intricate organ called the kidney.

Transparency

T-08-280-225-1



$$R - \overset{\displaystyle H \atop |}{\underset{| \atop \displaystyle NH_2}{C}} - COOH$$

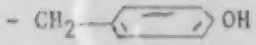
Carboxyl group

amine group

Amino acid: general form

R = one of 20 variable groups

Example:

R	amino acid
$- H$	glycine
$- CH_2 - SH$	cysteine
$- CH_2 - OH$	serine
	tyrosine

NOTE: Used in II A 2 a - d

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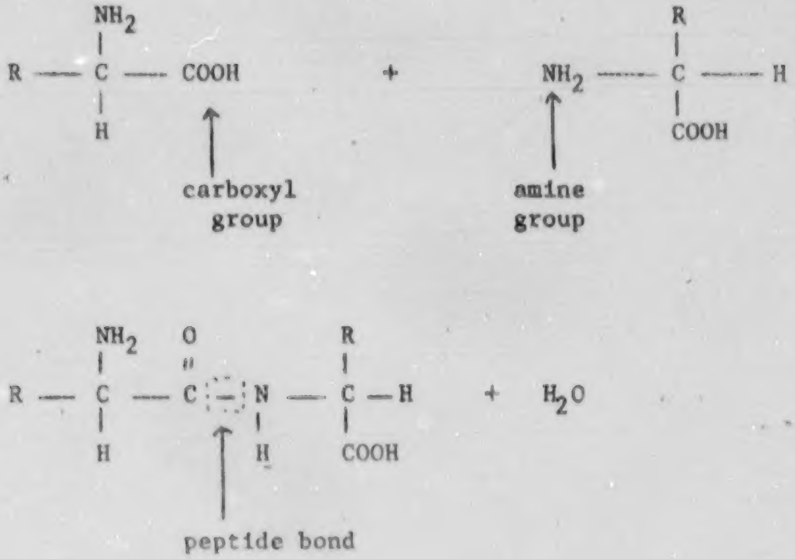
T - 08-280-225-2

Formation of Peptide Bond:


carboxyl group
amine group
peptide bond

$$R-\underset{H}{\overset{NH_2}{C}}-COOH + NH_2-\underset{COOH}{\overset{R}{C}}-H \longrightarrow R-\underset{H}{\overset{NH_2}{C}}-\overset{O}{\overset{\|}{C}}-\underset{H}{N}-\underset{COOH}{\overset{R}{C}}-H + H_2O$$

Chain may be extended in both directions

NOTE: Used in II - A - 3b

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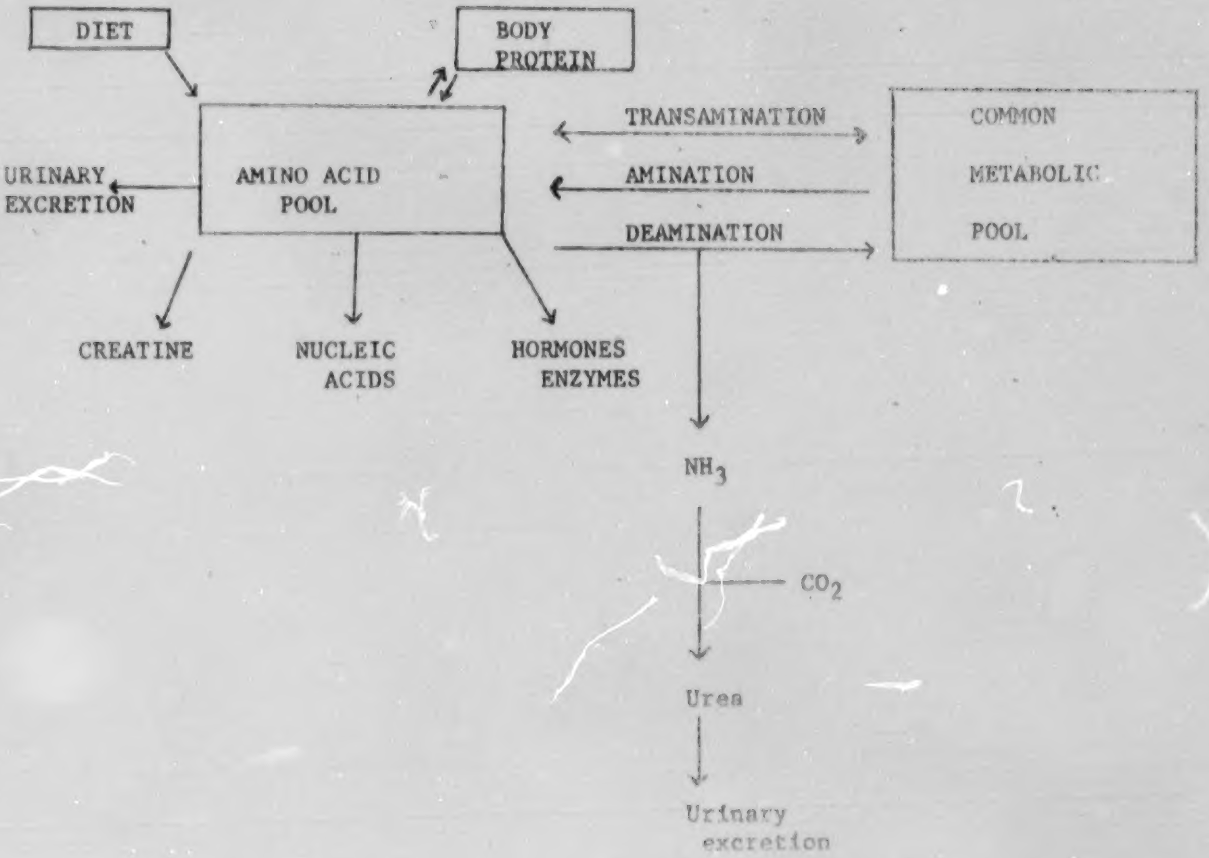
T - 08-280-225-3


DIET
BODY PROTEIN
AMINO ACID POOL
URINARY EXCRETION
CREATINE
NUCLEIC ACIDS
HORMONES ENZYMES
TRANSAMINATION
AMINATION
DEAMINATION
COMMON METABOLIC POOL
NH_3
CO_2
Urea
Urinary excretion

NOTE: Used in II - D - 5 a-c

From Ganong, W. F. Review of Medical Physiology, Los Altos, Calif.
7th Ed. p. 212

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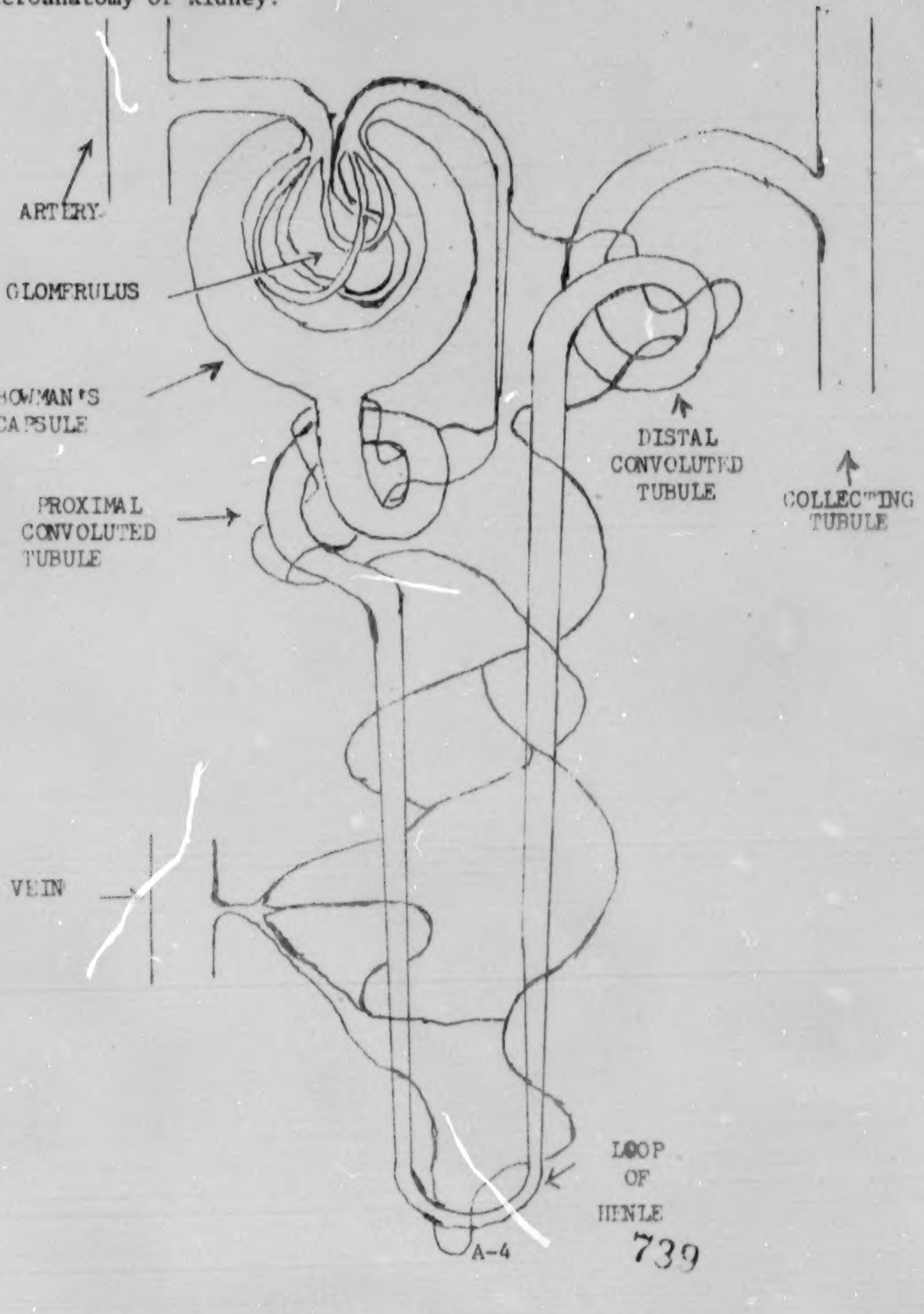
T 08-280-225-4 (as illustrated below)

Microanatomy of Kidney:


ARTERY
GLOMERULUS
BOWMAN'S CAPSULE
PROXIMAL CONVOLUTED TUBULE
DISTAL CONVOLUTED TUBULE
COLLECTING TUBULE
VEIN
LOOP OF HENLE

A-4

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T - 08-280-225-5

BUN Procedure: DAM-TSC METHOD

BUN + Diacetyl Monoxime $\xrightarrow[\text{acid, heat}]{\text{Thiosemicarbofide}}$

Pink color

Measured at 520nm

$$\frac{\text{Concentration of standard}}{\text{Absorbance of standard}} = \text{K factor}$$

Absorbance of unknown X K = concentration of unknown

NOTE: Used in II F 2 - G

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-225-2
LABORATORY SCIENCES DIVISION 084
CHEMISTRY BRANCH

Urea Nitrogen
(Diacetyl Monoxime - Thiosemicarbazide Method)

A. PRINCIPLE

Urea in serum or plasma reacts directly with diacetyl monoxime in a hot, acid environment to form a pink colored complex. The catalyst thiosemicarbazide intensifies the color. The intensity of the color is measured spectrophotometrically at 520 nm and is proportional to the concentration of the urea in the specimen.

B. PROCEDURAL NOTES

1. Although urea reacts directly in the test, values are reported as urea nitrogen.

2. The color's intensity for specimens with concentrations from 5 to 60 mg/dl follows Beer's law. For elevated test values, the procedure must be repeated with a diluted sample. The final reaction mixture cannot be diluted to bring its absorbancy into range.

3. Normal values and precision of the test are comparable to urease methods although, with elevated BUN values, discrepancies appear to be due to lesser specificity of the diacetyl monoxime method (i.e., values are 5 to 15 percent higher with the diacetyl monoxime than with urease methods when the BUN value exceeds 100 mg/dl).

4. The chief advantage of using the diacetyl monoxime method is that ammonium ions are not formed nor measured and will not act as a source of test contamination as with the urease methods.

5. Thiosemicarbazide, unlike other intensifiers of the color reaction, is water soluble and gives a non-photosensitive complex.

6. Ferric ions and the catalyst thiosemicarbazide increase the sensitivity of the color reaction and reduce the acid requirements to about one-tenth the amount needed without these reagents.

7. Solutions used in this procedure are the same as those with the AutoAnalyzer and both give results comparable to the urease

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*This mimeo supersedes M 08-280-300-1, 092.

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methods (the AutoAnalyzer method usually runs about one milligram per deciliter higher than the manual methods).

C. REAGENTS

1. Stock diacetyl monoxime solution, 2.5 g/dl (w/v). Place 25 g of diacetyl monoxime (2,3-butadione-2-oxime) into a one liter volumetric flask and add approximately 800 ml of deionized water. Swirl the contents of the flask until all the salts are dissolved and qs to the mark with deionized water. Store in a brown plastic bottle.

2. Stock thiosemicarbazide solution, 0.5 g/dl (w/v). Place 5 g of the thiosemicarbazide into a one liter volumetric flask and add approximately 800 ml of deionized water. Swirl the contents of the flask until all the salts are dissolved and qs to the mark with deionized water. Store in a plastic bottle.

3. Working BUN color reagent (diacetyl monoxime - thiosemicarbazide). In a one liter volumetric flask, mix 67 ml of the stock diacetyl monoxime solution and 67 ml of the stock thiosemicarbazide solution. QS to the mark with deionized water, mix thoroughly by inversion, and store in a brown plastic bottle.

4. Stock ferric chloride-phosphoric acid solution. Place 300 ml of concentrated phosphoric acid (H_3PO_4, 85 percent (w/w)), into a 500 ml graduated cylinder and add 15 g of hydrated ferric chloride ($FeCl_3 \cdot 6H_2O$). Swirl the contents of the cylinder until all the salts are dissolved and add sufficient deionized water to bring the total volume of the solution to 450 ml. (CAUTION: adding water to acid causes an extreme exothermic reaction. Add the water slowly with constant swirling.) Store in a brown glass bottle.

5. Stock sulfuric acid solution, 13 ml/dl (v/v). Slowly, with constant swirling, add 130 ml of concentrated sulfuric acid (H_2SO_4) to 800 ml of deionized water in a 2 liter Erlenmeyer flask. Swirl the contents of the flask with the flask in a cold water or ice bath to control the formation of excessive heat on mixing the two solutions. Cool to room temperature and store in a plastic bottle.

6. Working BUN acid reagent. Pipet 1.0 ml of the stock ferric chloride-phosphoric acid solution into a one liter volumetric flask and qs to the mark with the stock sulfuric acid solution. Mix thoroughly by inversion and store in a brown plastic bottle.

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7. Sulfuric acid solution, 0.01 N. Place approximately 1000 ml of deionized water into a two liter volumetric flask and slowly add 0.55 ml of concentrated sulfuric acid (H_2SO_4). Mix by swirling and qs to the mark with deionized water. Mix thoroughly by inversion and store in a plastic bottle.

8. Stock urea standard solution, 15 mg nitrogen/ml. Place 3.214 g of ACS grade urea (H_2NCONH_2) into a 100 ml volumetric flask. Add approximately 50 ml of 0.01 N H_2SO_4 and swirl the mixture until all salt is dissolved. QS to the mark with 0.01 N H_2SO_4 and mix thoroughly by inversion. Store in a plastic bottle.

9. Working urea nitrogen standard solution, 0.15 mg nitrogen/ml. Pipet 1.0 ml of the stock urea standard solution into a 100 ml volumetric flask and qs to the mark with 0.01 N H_2SO_4. Mix thoroughly by inversion and store in a plastic bottle.

D. SPECIMEN OF CHOICE

Serum or plasma

E. EQUIPMENT NEEDED

1. Heating block (95 to 100°C) or boiling water bath
2. 16 x 150 mm test tubes
3. 10 ml serological pipets
4. 0.02 ml micropipets
5. 19 x 150 mm (large) cuvets
6. Spectrophotometer

F. PROCEDURAL STEPS

1. Appropriately label 16 x 150 mm test tubes, one for each REAGENT BLANK, STANDARD, CONTROL, and UNKNOWN.

2. Pipet 3.0 ml of the BUN color reagent into each of the tubes. (Use a 10 ml serological pipet, line to line.)

3. Pipet 3.0 ml of the BUN acid reagent into each of the tubes. (Use a 10 ml serological pipet, line to line.)

4. Pipet each of the following solutions into the appropriate tube. (Use micropipets such as the Salhi pipet.)

   a. 0.02 ml of the working standard solution into the STANDARD tube.

   b. 0.02 ml of the control serum into the CONTROL tube.

   c. 0.02 ml of each unknown serum into its appropriate UNKNOWN tube.

5. The following chart is a summary of steps 2 through 4.

	BLANK	STANDARD	CONTROL	UNKNOWN(S)
BUN Color Reagent	3.0 ml	3.0 ml	3.0 ml	3.0 ml
BUN Acid Reagent	3.0 ml	3.0 ml	3.0 ml	3.0 ml
Working Standard Solution	------	0.02 ml	------	------
Control Serum	------	------	0.02 ml	------
Unknown Serum	------	------	-------	0.02 ml

6. Mix the contents of each tube thoroughly by inversion (use Parafilm).

7. Place all of the tubes into a boiling water bath (or heating block) for exactly 10 minutes.

8. Cool all of the tubes to room temperature (3-5 minutes in a cold water bath).

9. Transfer the contents of each tube into an appropriately labeled 19 x 150 mm cuvet.

10. Set the wavelength of the spectrophotometer to 520 nm and then, if necessary, adjust zero percent transmittance (0 %T).

11. Using the REAGENT BLANK cuvet, set one hundred percent transmittance (100 %T).

12. Read and record the absorbancy for each of the STANDARD, CONTROL, and UNKNOWN(S) cuvets.

G. CALCULATIONS

$$\frac{A_s}{C_s} = \frac{A_u}{C_u}$$

or

$$C_u = \frac{A_u}{A_s} \times C_s$$

WHERE: A_s is the absorbancy of the standard

C_s is the concentration of the standard (15 mg/dl)

A_u is the absorbancy of the control or unknown

C_u is the concentration of the control or unknown (as mg/dl)

H. NORMAL VALUES

5 to 25 mg/dl (as urea nitrogen)

I. CLINICAL SIGNIFICANCE

1. Decreased BUN levels are of little significance.
2. Increased BUN levels may be used as an index of kidney malfunction.

J. REFERENCES

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SP5 Bruce O. Woodall

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-230
PATHOLOGY AND LABORATORY SCIENCES DIVISION 084
CHEMISTRY BRANCH

Chloridometer (9 hours)

COURSES PRESENTED TO: 311-92B20, Medical Laboratory Procedures (Basic)

PLACE: Classroom/laboratory

REFERENCES:

Buchler Instruments. Instructions for Operation, Buchler-Cotlove Chloridometer. Fort Lee: Buchler Instruments Division, G. D. Searle and Company, 1969.

Seligson. Standard Methods of Clinical Chemistry. Vol III. New York: Academic Press, 1961, p 81.

Tietz, N. W., ed. Fundamentals of Clinical Chemistry. Philadelphia: W. B. Saunders Company, 1970, pp 621-625.

STUDY ASSIGNMENT: After class, review M 08-280-230-1 and -3 and study lecture notes.

STUDENT UNIFORM AND EQUIPMENT: Laboratory notebook

TOOLS, EQUIPMENT, AND MATERIALS: M 08-280-230-2 issued one per student at the beginning of the 1st hour. M 08-280-230-1 and -3 issued one each per student at the end of the 1st hour.

PERSONNEL: One instructor (MOS 92B4H) 1st and 9th hours; eight instructors (MOS 92B4H) 2d through 8th hours

INSTRUCTIONAL AIDS: Chalk, chalkboard, eraser, overhead projector and screen, T 08-280-230-1 through -4 (see ANNEX B), VT #570, and TV receivers (see ANNEX C)

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: L (1st hour), PE (2d thru 8th hours), EX (9th hour)

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*This lesson plan supersedes LP 08-280-320, 081.

LP 08-280-230
084

FIRST HOUR

NOTE: Issue M 08-280-230-2, one per student. Students take lecture notes on this mimeo.

I. INTRODUCTION (5 min)

A. Opening Statement: The importance of measuring serum and cerebrospinal fluid chloride concentrations has a historical basis. Chloride was the first of the electrolytes that could easily be measured. Thus, a long clinical and laboratory experience was established with the chloride ion before reliable measurements for sodium and potassium were available.

B. Objectives

1. State the principles involved in the use of the Chloridometer.

2. List the procedural steps for the preparation, blanking, standardization, and use of the Chloridometer.

3. State the normal value ranges for serum and cerebrospinal fluid chloride concentrations.

4. With practice, satisfactorily quantitate serum and cerebrospinal chloride concentrations using the Chloridometer.

C. Class Procedure and Lesson Tie-in

1. First hour of nine on the Chloridometer

2. To be followed by a practical exercise in the quantitation of chloride concentrations and an examination on the material presented.

II. EXPLANATION (35 min)

A. Principles of the Chloridometer

1. Electrodes generate silver ions

a. Silver ions released

b. Fixed quantity per second

2. Chloride ions unite with silver ions

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a. Undissociated AgCl (precipitate)

b. All chloride ions united (used up)

NOTE: See ANNEX A for the complete reaction equations.

3. Endpoint is reached

a. Excess silver ions

b. Increase of conductivity

c. Stopping of titration

NOTE: Show T 08-280-230-1 thru -3 to outline reactions in the vial (see ANNEX B).

4. Readout (direct)

a. Titration time proportional to chloride concentration

b. Unit of report - mEq/L

QUESTION: Why do we use mEq/L as the unit of report for serum chloride concentrations?

ANSWER: mEq/L is an expression of reacting strength of the solution and the reporting units are whole numbers since the chloride concentration is small.

B. Preparation of the Chloridometer

1. Adjust length of electrodes (if necessary)

NOTE: If the generator electrode becomes thinner than a household pin, it should be cut and adjusted to the proper length.

2. Clean all electrodes

a. Silver polish

b. Deionized water

3. Turn ON/OFF switch to ON position

NOTE: When the instrument is turned ON, the stirrer begins to turn. The ADJUST/TITRATE switch is in the TITRATE position. To stop the titration cycle, turn the ADJUST/TITRATE switch to ADJUST, then back to TITRATE and move the red pointer of the microammeter to meet the black pointer. This stops the titration cycle.

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4. Turn the TITRATION RANGE switch to HIGH

5. Warm up for 5 to 10 minutes

6. When determinations are completed, turn ON/OFF switch to OFF

C. Procedures for Use of the Chloridometer

1. Blanking

a. Blank solution

(1) Pipet into titration vials

(a) 4.0 ml of acid diluent

__QUESTION__: What type and size of pipet should be used to pipet the acid diluent?

__ANSWER__: A 5 or 10 ml serological pipet, line to line.

(b) Four drops of gelatin reagent

(2) Prepare four vials

__NOTE__: Show T 08-280-230-4 to point out the items of importance on the face of the Chloridometer (see ANNEX B).

b. Place blank vial into vial holder

c. Place DIRECT READER switch to BLANK

__NOTE__: Digital readout should read 0000.

d. Raise the vial onto titrate position

e. Turn ADJUST/TITRATE switch to ADJUST

(1) Black pointer should drop to below 5 microamperes

(2) Pointer should stop dropping within thirty seconds

(3) Adjust red pointer to ten microamperes above the black pointer

f. Turn ADJUST/TITRATE switch to TITRATE

g. Titration takes place

NOTE: The number appearing on the digital readout will be the blanking time in tenths of seconds.

h. Repeat the blanking three times

NOTE: After cleaning the electrodes, it may take several blanking cycles to condition the electrodes before a consistent blank time is obtained.

i. Calculate the average blank time

j. Set the average blank time on the BLANK TIMER

NOTE: The blank time is set by turning the white pointer until it reads the correct blanking time.

2. Standardizing

a. Standard solution

(1) Pipet into titration vial

(a) 4.0 ml of acid diluent

(b) 0.1 ml of standard (100 mEq/L)

QUESTION: What type and size of pipet should be used to pipet the standard solution?

ANSWER: A 0.2 ml micropipet.

(c) Four drops of gelatin reagent

(2) Prepare four vials

b. Place standard vial into vial holder

c. Place DIRECT READER switch to TITRATE

NOTE: Digital readout should be set to 0000.

d. Raise the vial into titrate position

e. Turn ADJUST/TITRATE switch to ADJUST

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(1) Black pointer should drop to below 5 microamperes

(2) Adjust red pointer to ten microamperes above the black pointer

f. Turn ADJUST/TITRATE switch to TITRATE

g. Titration takes place

h. Read chloride concentration

QUESTION: What is the unit of report for chloride concentrations in serum?

ANSWER: mEq/L

NOTE: The number appearing on the digital readout is the concentration of chloride ions in the standard solution as mEq/L.

i. Repeat the standardizing three times

NOTE: The allowable range for the standard reading is 98 to 102 mEq/L.

3. Testing unknowns

a. Unknown solution

(1) Pipet into titration vial

(a) 4.0 ml of acid diluent

(b) 0.1 ml of the unknown serum

(c) Four drops of gelatin reagent

(2) Prepare one vial for each unknown

b. Test in the same manner as the standardizing procedure

NOTE: The reading from the digital readout is the chloride concentrations of the unknowns as mEq/L.

D. Normal Values

1. Serum - 98 to 106 mEq/L

2. CSF - 123 to 128 mEq/L

751

732

LP 08-280-230
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NOTE: Point out that the chloride concentrations in cerebrospinal fluid are normally higher than those in serum.

NOTE: Issue M 08-280-230-1 and -3, one each per student.

E. Questions From Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Principles of the Chloridometer
2. Preparation of the Chloridometer
3. Procedures for use of the Chloridometer
4. Normal values

B. Closing Statement: It is very important for the medical laboratory specialist to accurately report the chloride values to the physician. These values can be used by the physician in the diagnosis of an acid-base disorder within a patient. The Chloridometer can help you to quickly and accurately report these values.

SECOND THROUGH EIGHTH HOURS

IV. APPLICATION (305 min)

A. Videotape (25 min) (see ANNEX C)

1. Introduction

   a. The Chloridometer (19:50 min)

   b. Points to be observed

      (1) Principles

      (2) Components

      (3) Reactions

      (4) Operating procedures

      (5) Sample preparation

2. Showing of videotape #570

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3. Discussion and questions

B. Instructors' Activities

1. Step by step, orient the students to the use, care, and maintenance of the Chloridometer.

2. Provide specimens and reagents.

3. Supervise students' activities.

4. Make on-the-spot corrections of students' errors.

5. Direct the students in correcting out-of-control test values.

6. Collect and grade students' report sheets.

C. Students' Activities

1. Become familiar with the use, care, and maintenance of the Chloridometer.

2. Satisfactorily perform chloride determinations using the Chloridometer.

3. Maintain proper records.

   a. Laboratory notebook

   b. Quality control chart

4. Obtain directions from the instructors for correction of out-of-control test values.

5. Turn in report sheet to the instructors.

6. Clean area and equipment as required.

V. CRITIQUE (10 min)

A. Questions From Students

NOTE: This videotape #570 is available for student viewing in the Learning Resource center.

B. Review of Main Points

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1. Technique

2. Sources of error

C. Closing Statement: All laboratory procedures must be performed with care and precision. The Chloridometer, when properly used, can give you quick and accurate test results for chloride concentrations to aid the physician in his diagnosis and treatment of his patients.

NINTH HOUR

VI. EXAMINATION (45 min)

A. Directions to Students

1. Put away all lecture notes and text references.

2. M 08-280-205-1 and -3 may be used.

B. Instructor's Activities

1. Issue quiz sheet, one per student.

2. Supervise students' activities.

3. Clarify any vague points.

4. Collect and grade completed quiz sheets.

C. Students' Activities

1. Place identification on quiz sheet.

2. Answer all questions as appropriate.

3. Turn in completed quiz sheet to the instructor.

D. Critique

1. Questions from students

2. Reteach instructional material as necessary.

INSTRUCTIONAL HOURS

Number of instructors required - - - 8

Primary hours	9
Secondary hours	49
Total	58

ANNEX A
INSTRUCTIONAL AIDS

Chemical Reactions

$$Ag^{+} + Cl^{-} \longrightarrow AgCl\downarrow$$

$$\underset{\text{(excess)}}{Ag^{+}} + AgCl\downarrow \longrightarrow AgCl\downarrow + Ag^{+}$$

(no Cl^- to unite with the Ag^+)

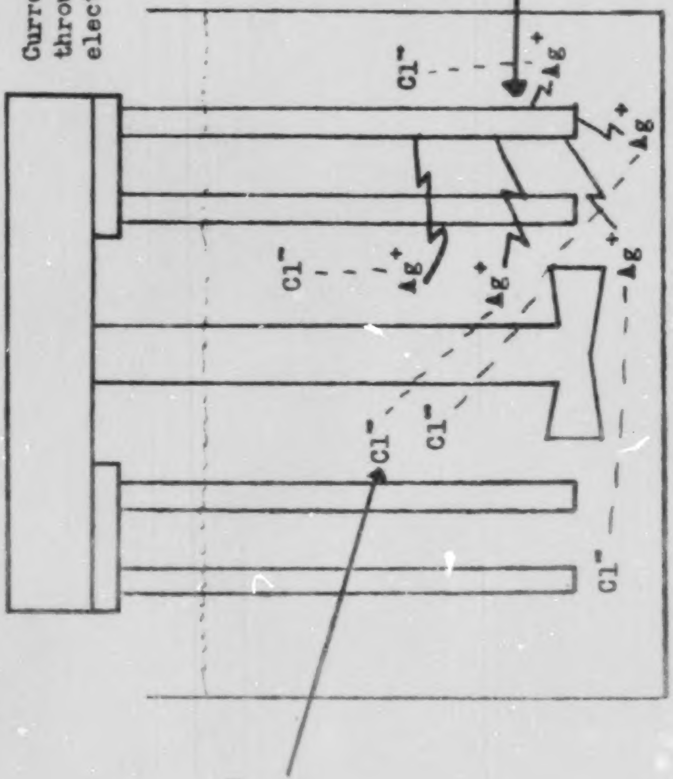
(excess Ag^+ cause an increase in conductivity)

NOTE: Used in part IIA? during the first hour.

ANNEX B
INSTRUCTIONAL AIDS

Transparencies

T 08-280-230-1 (as illustrated below).



Current is passed through silver electrode.

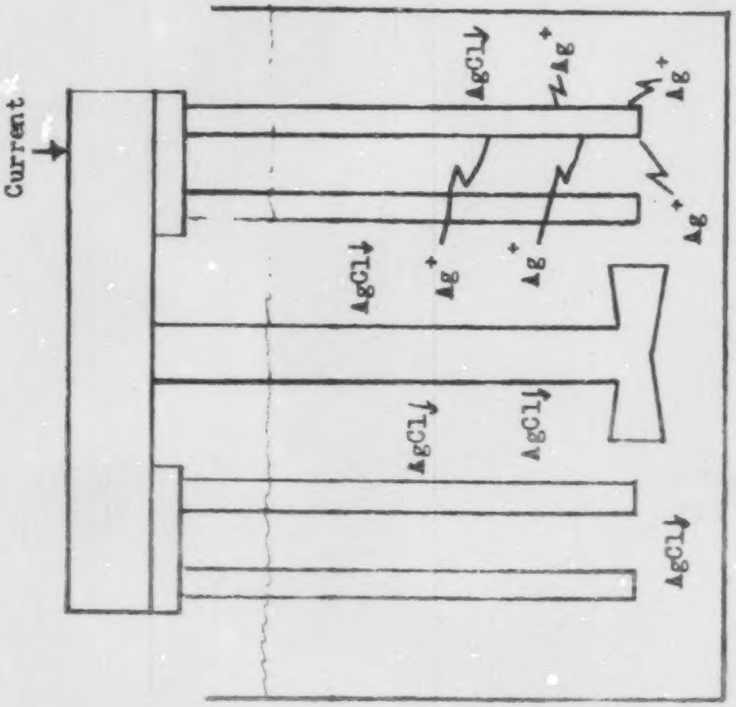
Silver ions being released because of electric current.

Chloride ions present from specimen.

Silver ions and chloride ions are attracted to each other.

NOTE: Used in part IIA during the first hour.

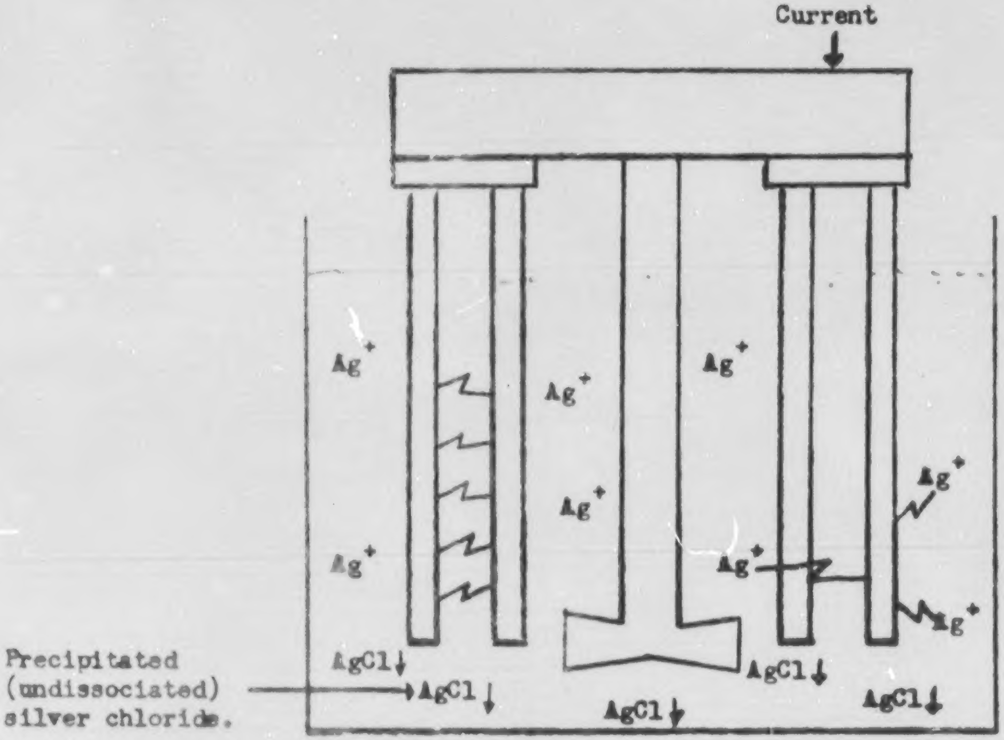
T 08-280-230-2 (as illustrated below).


Current
AgCl↓
AgCl↓
AgCl↓
AgCl↓
AgCl↓
Ag+
Ag+
Ag+
Ag+
Ag+
Ag+

All of the available chloride ions combine with silver ions. An excess of silver ions accumulate.

NOTE: Used in part IIA during the first hour.

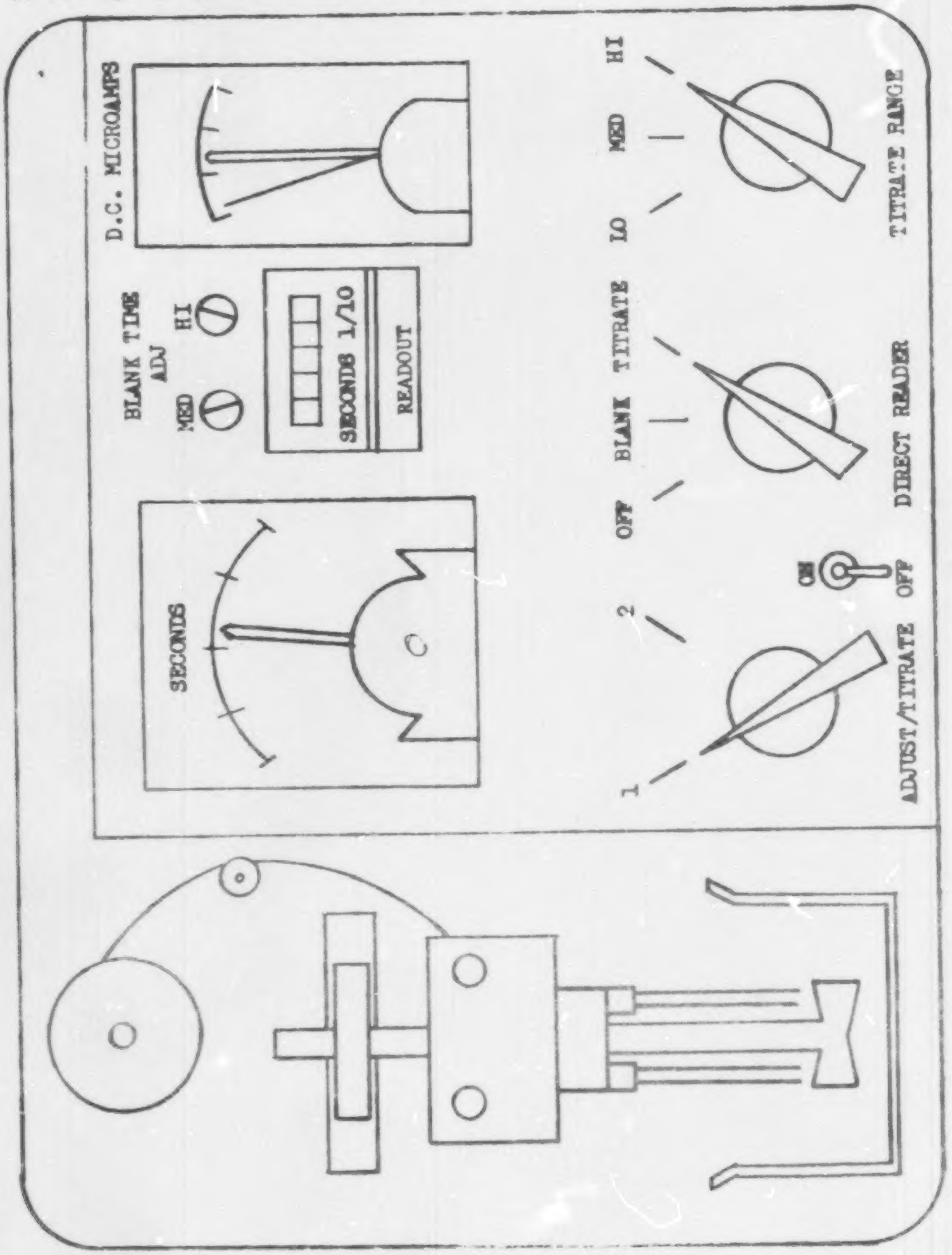
T 08-280-230-3 (as illustrated below).


Current
Ag+
Ag+
Ag+
Ag+
Ag+
Ag+
Ag+
Ag+
Precipitated (undissociated) silver chloride.
AgCl↓
AgCl↓
AgCl↓
AgCl↓
AgCl↓

Surge of conductivity caused by the excess of silver ions triggers switch to signal endpoint of titration.

NOTE: Used in part IIA during the first hour.

T 08-280-230-4 (as illustrated below).


D.C. MICROAMPS
BLANK TIME
ADJ
MED
HI
SECONDS 1/10
READOUT
SECONDS
1
2
OFF
BLANK
TITRATE
LO
MED
HI
ON
OFF
ADJUST/TITRATE
DIRECT READER
TITRATE RANGE

NOTE: Used in part IIC during the first hour.

SP5 Bruce O. Woodall

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *IM 08-280-230
PATHOLOGY AND LABORATORY SCIENCES DIVISION 084
CHEMISTRY BRANCH

Chloridometer

The importance of measuring serum and cerebrospinal fluid chloride concentrations has a historical basis. Chloride was the first of the electrolytes that could easily be measured. Thus, a long clinical and laboratory experience was established with the chloride ion before reliable measurements for sodium and potassium were available.

The instrument used to determine chloride concentrations of specimens is the Chloridometer. The Chloridometer operates on the principle of silver ions being released at a fixed quantity per second as a result of an electric current passing through the silver (generator) electrodes. These silver ions then combine with free chloride ions in the test solution to form undissociated silver chloride. When all free chloride ions have been united with the silver ions, the excess of silver ions produced by the generator electrodes remain "free" in the solution causing a rapid increase in conductivity of the solution. This increase of conductivity is detected by the indicator electrode which then signals a relay switch to terminate the titration. This is the "end point" of the titration and the direct reader shows the concentration of chloride ions in the specimen as milliequivalents per liter (or as the seconds it takes to make the titration).

As with any instrument in the clinical laboratory, there are certain necessary steps to take in order to keep the instrument as accurate as possible. We must first insure that the electrodes are clean and of proper length and thickness. If the generator electrode becomes thinner than a household pin, it should be cut and adjusted to the proper length. Silver polish is used to remove the tarnish and other impurities from the surface of the electrodes and stirrer. After cleaning, the electrodes and stirrer should be rinsed well with deionized water.

When using the Chloridometer, the instrument's power source is turned on and the titration range turned to high for testing serum or cerebrospinal fluid specimens. (When the instrument is turned ON, the stirrer begins to turn and the titration cycle activated. To stop the titration cycle, turn the ADJUST/TITRATE switch to ADJUST, then back to TITRATE and move the red pointer of the microammeter to meet the black pointer. This stops the titration cycle.) Let the instrument warm up for five to ten minutes before running any tests.

Before any unknowns are run on the Chloridometer, it is necessary to blank and standardize the instrument to insure accuracy of test results.

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*This instructor's manuscript supersedes IM 08-280-320, 081.

Generally, the Chloridometer should be blanked and standardized daily before any tests are run. Blanking the Chloridometer records, in seconds, the time taken for the indicator electrodes to detect the increase in conductivity in the blank solution (a combination of acid reagent and gelatin reagent). With the blanking procedure, we can negate that time so that the readout of the standards and unknown specimens represents only the concentration of chloride in the standard or unknown solutions.

The procedure for blanking the Chloridometer begins with the preparation of the blank solutions. The blank solutions contain four milliliters (4 ml) of the acid diluent and four (4) drops of the gelatin reagent. At least four (4) vials of blank solutions should be prepared. Insure that the electrodes are rinsed with deionized water (before the first and between each succeeding titration) and place a blank vial into the vial holder. Turn the direct reader switch of the Chloridometer to the blank position and insure that the digital readout reads zero (0000). Raise the blank vial into titration position, making sure that the electrodes are fully immersed, and turn the ADJUST/TITRATE switch to the ADJUST position. At this time, the black pointer of the microammeter should drop to a position somewhere below five (5) microamperes. When the pointer has dropped to its lowest point (within thirty seconds), turn the red pointer of the microammeter to ten (10) microamperes above the black pointer. Turn the ADJUST/TITRATE switch to the TITRATE position, starting the titration. Record the blank time from the readout and repeat the blanking procedure with the remaining blank vials. (After cleaning the electrodes with silver polish, it may take several blank titrations to condition the electrodes before a consistent blank time is obtained.) Calculate the average blank time and set the blank timer appropriately by turning the white pointer of the blank timer to the correct blank time.

Once the Chloridometer is properly blanked, it is necessary to standardize the instrument. The standard is used as a reference to insure the accuracy of the instrument's calibration. Standard solutions contain four milliliters (4 ml) of the acid diluent, one-tenth milliliter (0.1 ml) of the standard solution, and four (4) drops of the gelatin reagent. Prepare at least four (4) vials of the standard solutions. After the electrodes have been rinsed with deionized water, place a vial of the standard solution into the vial holder and raise it into titrate position. With the digital readout registering zero (0000), turn the direct reader switch to titrate position and the ADJUST/TITRATE switch to ADJUST position and allow the black pointer of the microammeter to drop to its lowest position. When the black pointer of the ammeter has stopped dropping, set the red pointer to a position ten microamperes above the black pointer. Turn the ADJUST/TITRATE switch to TITRATE position. The titration will now take place. When the titration stops, record the chloride concentration, as milliequivalents per liter, from the readout. Repeat the standardization procedure with the remaining standard vials and, if necessary, calibrate the instrument using the standard adjust screw located directly above the direct readout panel. The allowable range for a one hundred milliequivalent per liter (100 mEq/L) standard is ninety-eight to one hundred and two milliequivalents per liter (98 to 102 mEq/L) reading on the readout panel.

Once we have the Chloridometer standardized, we are ready to test the unknown specimens. The unknowns are diluted in a manner similar to that used for the standards; i.e., four milliliters (4 ml) of the acid diluent, one-tenth milliliter (0.1 ml) of the unknown, and four (4) drops of the gelatin reagent are mixed. Test the vials of unknowns (one test per unknown) in the same manner as the standards. The readout will record the chloride concentrations of the specimens as milliequivalents per liter. When the tests are completed, simply turn off the Chloridometer's power source.

The normal values range for chloride concentrations in serum is ninety-eight to one hundred and six milliequivalents per liter (98 to 106 mEq/L) and one hundred twenty-three to one hundred twenty-eight milliequivalents per liter (123 to 128 mEq/L) for cerebrospinal fluid. Note that the chloride concentrations found in cerebrospinal fluid are normally higher than those in serum.

During this period of instruction, we have discussed the principles of the Chloridometer, how to prepare the Chloridometer for use (blanking, standardizing, and running specimens), and the normal values of chloride concentrations in serum and cerebrospinal fluid.

It is very important for the medical laboratory specialist to accurately report the chloride values to the physician. These values can be used by the physician in the diagnosis of an acid-base disorder within a patient. The Chloridometer can help you to quickly and accurately report these values.

FIRST HOUR

NOTE: Issue M08-280-230-1, one per student. At beginning of class

I. INTRODUCTION (5 minutes)

- A. Opening Statement: The importance of measuring serum and CSF chloride concentrations has a historical basis. Chloride was the first of the electrolytes that could easily be measured. Thus, a long clinical and laboratory experience was established with the chloride ion before reliable measurement for sodium and potassium were available.
- B. Objectives
    1. Select the principles involved in the use of the Chloridometer
    2. Select the normal value ranges for serum and CSF chloride concentrations.
    3. Select the chief anion in the extracellular fluid.
    4. With practice, satisfactorily quantitate serum and CSF chloride concentrations.
- C. Class Procedure and Lesson Tie-in
    1. First hour of eleven on Chloride.
    2. To be followed by a practical exercise in the quantitation of chloride concentrations.

II. EXPLANATION (35 minutes)

- A. Physiology
    1. Chief extracellur anion
    2. Absorbed in small intestine
    3. Whole blood concentration regulated by kidneys (glomerular filtration - reabsorbed by proximal tubules)
    4. Function
        - a. Used to produce HCI in gastric fluid
        - b. Used in an intermediate step in removal of CC_2
        - c. Aid in acid - base balance
        - d. Aid in electrolyte balance
        - e. Enzyme activator - amylase

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B. Clinical Significance

1. Normal values
   a. Serum 98 - 106 mEq/L
   b. CSF 123 - 128 mEq /L

2. Increased values
   a. Dehydration
   b. Congestive heart failure - decreased blood flow thru the kidneys

3. Decreased values
   a. Vomiting/diarrhea
   b. Kidney disease - deficient tubular reabsorption
   c. CNS disease - meningitis (in CSF)

NOTE: Dehydration caused by vomiting and/or diarrhea may have opposite effects on chloride concentration

C. Laboratory Methods

1. Colorimetric - AA

2. Ion specific electrode

3. Titimetric
   a. Schales and Schales - indicator (Diphenylcarbazone)
   B. Coulometric - Chloridometer

QUESTION: What is the unit of report for chloride concentrations in serum?

Answer: mEq/L

D. Chloridometer

1. Principle
   a. Electrode generates silver ions at fixed rate
   b. Chloride unites with silver ions

      (1) AgCl precipitates
      (2) Excess silver ions generated

   c. Endpoint reached

      (1) Increase in conductivity
      (2) Titration stops
      (3) Titation time proportional to chloride concentration
      (4) Direct readout, reported in mEq/L

NOTE: Show T 08-280-230-1 thru -3 to outline reactions in the vial (see ANNEX A)

2. Specimen testing
   a. Unknown and control preparation
      (1) 4.0 ml. of acid diluent
      (2) 100 λ of specimen or control
      (3) 4 drops of gelatin reagent

NOTE: Chloridometer will be blanked and standardized by instructors

   b. Titration
      (1) Turn adjust/titrate switch to adjust position
         (a) Black pointer must drop below 5 microampers
         (b) Adjust red pointer for ten microampers above black pointer
      (2) Turn adjust/titrate switch to titrate position
      (3) Record results as direct readout in mEq/L
      (4) Rinse electrodes with distilled water
      (5) Run remaining specimens as above

QUESTIONS: What causes the completion of titration?

ANSWER: Excess silver ions present which increase conductivity.

E. Questions from Students

III. SUMMARY (5)

A. Review of Main Points

1. Physiology of chloride

2. Clinical significance

3. Laboratory methods for chloride determinations

4. Principles and use of Chloridometer

B. Closing Statement: It is very important for the medical laboratory specialist to accurately report the chloride values to the physician. These values can be used by the physician in the diagnosis of an acid - base disorder within a patient. The Chloridometer can help you to quickly and accurately report these values.

SECOND THROUGH ELEVENTH HOURS

IV. APPLICATION (440 minutes)

A. Instructors' Activities

1. Step by step, orient the students to the use, care, and maintenance of the Chloridometer.

2. Provide specimens and reagents.

3. Supervise students' activities.

4. Make on-the-spot corrections of students' errors.

5. Direct the students in correcting out-of-control test values.

6. Collect and grade students' report sheets.

B. Students' Activities

1. Become familiar with the use, care, and maintnance of the Chloridometer.

2. Satisfactorily perform chloride determinations using the Chloridometer.

3. Maintain proper records
    a. Laboratory notebook
    b. Quality control chart

4. Obtain directions from the instructors for correction of out-of-control test values.

5. Turn in report sheet to the instructors.

6. Clean area and equipment as required.

V. REVIEW (10 minutes)

A. Questions From Students

B. Review of Main Points

1. Technique

2. Sources of error

C. Closing Statement: All laboratory procedures must be performed with care and precision. The Chloridometer, when properly used can give you quick and accurate test results for chloride concentrations to aid the physician in his diagnosis and treatment of his patients.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-230-1
LABORATORY SCIENCES DIVISION 063
CHEMISTRY BRANCH

CHLORIDE TITRATIONS

The Buchler-Cotlove Chloridometer

A. PRINCIPLES

The generator electrodes of the chloridometer produce silver ions at a fixed quantity per second. Chloride ions combine with these silver ions to form undissociated silver chloride. The endpoint of the titration occurs when no more chloride ions are present in the titrant, and the silver ions produced by the generator electrode remain "free" in solution. Indicator electrodes record this rapidly increasing conductivity of the titrant solution, and signal a realy switch to "shut down" the digital recorder and generator electrode circuitry.

The titration is a COULOMETRIC titration, since a quantity of electricity in Coulombs (Q) is measured at a fixed potential during a specific time. The number of Coulombs (Q) generated is equal to the product of the current (I) times the time (T). We say that:

$$Q = I \times T$$

and $Q = \boxed{I} \times T$

when

$\underline{I}$ is kept constant: Q is proportional to T.

The time necessary to reach the endpoint is taken as a relative measure of the chloride concentration in a given sample. The titration time of a standard is to its known concentration as the titration time of an unknown is to its concentration. The ratio established allows us to calculate the concentration of chloride (in mEq/1.) in control and unknown samples.

Once standardized, the Buchler-Cotlove Chloridometer establishes the aforementioned ration electronically so that unknowns may be read accurately within about one minute titration time.

B. REAGENTS

1. Acid Diluent Reagent

to 900 ml of chloride free water, add 6.4 ml of concentrated nitric acid and 100 ml glacial acetic acid (reagent grade). This reagent is stable for one year at room temperature.

2. Dry Gelatin Reagent (Stock)

Thoroughly mix 3.00 g unflavored gelatin with 0.05 g thymol crystals (reagent grade), and add 0.05 g thymol blue [water soluble].

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*This mimeo supersedes M 08-280-320-2, 092

3. Gelatin Reagent (Working)

To 3.1 g of the dry gelatin reagent (stock) add approximately 500 ml of chloride free water, heating gently until the solution is clear (swirl continuously). Dispense into small screw cap vials which have been rinsed with chloride-free water. Place approximately 5 ml of reagent in each vial and refrigerate (DO NOT FREEZE). The refrigerated reagent is stable for about 6 months. To use: Immerse the tube in warm water until the reagent liquifies. Discard excess reagent at end of each day's run.

4. Chloride Standard (100 mEq/l.)

Dissolve 5.845 g. of dessicated NaCl in a 1000 ml volumetric flask which has been rinsed with chloride free water. Add chloride free water (ONLY) and q.s. to 1000 ml. Store at room temperature in a tightly stoppered polyethylene bottle. CAUTION: Keep bottle well-stoppered when not in use since evaporation will significantly change the standard concentration.

C. SAMPLE PREPARATION

1. Blank (Prepare 4)

   a. Deliver 4.0 ml of acid diluent to clean glass vials.

   b. Add four drops of gelatin reagent (working) to each vial.

   c. Cover vial with Parafilm.$^{(R)}$

2. Standard (100 mEq/l.) (Prepare 4)

   a. Deliver 4.0 ml of acid diluent to clean glass vial.

   b. Deliver 0.1 ml of chloride standard (100 mEq/l.) to each vial with a micropipet. BE SURE TO USE PROPER RINSE-OUT TECHNIQUE.

   c. Add four drops of gelatin reagent (working) to each vial.

   d. Cover vial with Parafilm.$^{(R)}$

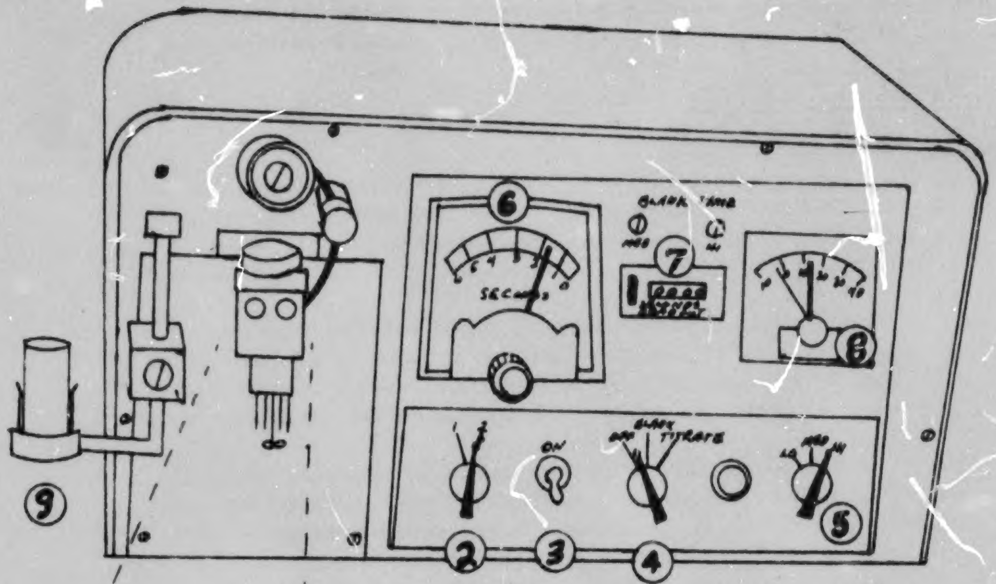
3. Control and Unknown (Prepare at least one control)

   a. Deliver 4.0 ml of acid diluent to clean glass vial.

   b. Deliver o.1 ml of control and each unknown to separate clean glass vials with a micropipet. BE SURE TO USE PROPER RINSE-OUT TECHNIQUE.

   c. Add four drops of gelatin reagent (working) to each vial.

   d. Cover with Parafilm.$^{(R)}$


6
SECONDS
BLANK TIME
7
8
ON
BLANK
TITRATE
9
2
3
4
5

(R)

BUCHLER-COTLOVE CHLORIDOMETER

1A, GENERATOR ELECTRODES

2A, INDICATOR ELECTRODES

2. ADJUST/TITRATE SWITCH

3. ON/OFF SWITCH

4. DIRECT READER SWITCH

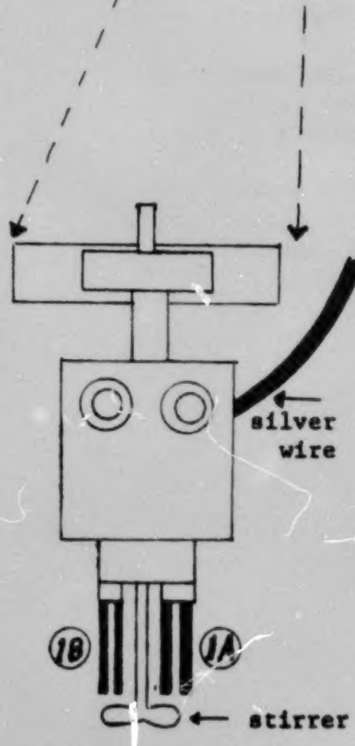
5. TITRATION RANGE SWITCH

6. BLANK TIMER

7. DIGITAL READOUT

8. DIRECT CURRENT AMMETER (Readings in Microamperes)

9. VIAL HOLDER ASSEMBLY


silver wire
1B
1A
stirrer

750

D. TITRATION PROCEDURE

NOTE: Refer when necessary to diagram of Buchler-Cotlove Chloridometer on page 3.

1. Preparation Of The Instrument

   a. Turn ON/OFF switch to "on" position.

   b. Set TITRATION RANGE SWITCH TO "High" position.

   c. Place machine in STAND-BY status:

      (1) Turn ADJUST/TITRATE SWITCH to"Position 2".

      (2) Bring adjustable pointer of DIRECT CURRENT AMMETER to meet indicator pointer. A distinct "click" is heard as relay places instrument in "stand-by" status.

   d. Clean ALL surfaces of electrodes.

      (1) Use only a water soluble silver polish.

      (2) Gently scrub tranish and residue from electrodes using a clean tissue, silver polish and a small amount of chloride-free water.

      (3) Rinse electrodes thoroughly with chloride-free water. NOTE: Water droplets remaining from this wash do NOT affect the titration. Do NOT attempt to dry electrodes. Inevitably you will CONTAMINATE the electrode surfaces.

2. Blanking Procedure

   a. Place vial containing blank in VIAL HOLDER ASSEMBLY.

   b. Turn DIRECT READER SWITCH to "blank."

   c. Clear DIGITAL READOUT DISPLAY be depressing the reset bar (located to immediate left of the display meter).

   d. Raise vial until ELECTRODES (1A and 1B) are immersed in the titrant.

   e. Turn ADJUST/TITRATE SWITCH to position one (1).

   f. Watch as indicator pointer on DIRECT CURRENT AMMENTER drops to its lowest reading (less than 5 microamperes).

   g. Adjust pointer on DIRECT CURRENT AMMETER ten divisions (10 microamperes) from the LOWEST or BASE reading. (i.e. Indicator falls to 3 microamperes - set adjustable pointer to 13 microamperes)

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h. Turn ADJUST TITRATE SWITCH to position two (2).

i. At the endpoint the distinct "click" signals--record the reading of the blank time (in tenths of seconds) from the DIGITAL READOUT DISPLAY.

j. Repeat this procedure (steps "a" through "i") with the 3 remaining blanks.

k. Average the blanking times and set the BLANK TIMER to this calculated average by turning the timer's adjustment knob until the indicator needle registers the value in tenths of seconds.

l. The machine is now "blanked." Wash electrodes between samples!

3 Standardization Procedure (100 mEq/l. chloride standard)

a. Place vial containing the 100 mEq/l. standard into the VIAL HOLDER ASSEMBLY.

b. Turn DIRECT READER SWITCH to "titrate."

c. REPEAT STEPS 2c. through 2l. of the aforementioned blanking procedure. REMEMBER: Wash electrodes between samples!

NOTE: Readings of the chloride standard are now expressed in milliequivalents per liter (mEq/l.) Acceptable results for standardization are from 99 to 101 mEq/l. chloride.

d. Repeat standardization procedure for the 3 remaining standards.

e. If standards read correctly the instrument is ready to titrate control and unknown samples. If readings for the standard are not uniform or if they are out of the acceptable limits of acceptability--call an instructor. Do NOT attempt to adjust the machine yourself.'

4. Control and Unknown Titrations

a. Follow same procedure as outlined above for standardization with each control and unknown sample.

b. The DIGITAL READOUT DISPLAY will record all concentrations in mEq/l.

E. CALCULATIONS None.

F. NORMAL VALUES

Serum chloride: 98 - 106 mEq/1.
Cerebrospinal fluid: 123 - 128 mEq/1.
Urine: 58 - 250 mEq/24 hr.

G. NOTES

1. The chloride content of all biological fluids can be obtained by this method. This includes: serum, urine, cerebrospinal fluid, whole blood, transudates, exudates, sweat, and gastro-intestinal fluid.

2. As with any method involving the reaction of chloride with the silver or mercuric ion, this method measures (in addition to chloride) other silver ion-combining substances such as: bromide, iodide, thiocyanate, sulfide or sulfhydril ions.

3. The chloride content of urine may vary from less than 1 mEq/1. to over 300 mEq/1. The reliability of values obtained decreases for urine concentrations less than 10 mEq/1., unless a special lower rate of titration is employed. Consult the Buchler-Cotlove Chloridometer Instruction Manual for this procedure.

4. Avoid reagent and specimen contact with rubber. Stoppers, rubber tubing, and rubber fittings on autodilutors may be a source of sulfhydril groups which contaminate the test.

5. Do NOT titrate a sample unless both gelatin and acid are present. The pink color (thymol blue at low pH) indicates the presence of acid diluent. In the absence of both gelatin and acid in the reagent diluent, the electrodes will become severely impaired.

H. BIBLIOGRAPHY

Instruction Manual for the Buchler-Cotlove Chloridometer$^{(R)}$, Fort Lee: Buchler Instruments Division of Searle and Co.

SFC Stephen E. Farabaugh

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-240
PATHOLOGY AND LABORATORY SCIENCES DIVISION 084
CHEMISTRY BRANCH

Practical Examination and Reteaching Session (9 hours)

COURSE PRESENTED TO: 311-92B20, Medical Laboratory Procedures (Basic)

PLACE: Classroom/laboratory

REFERENCES: All previous references

STUDY ASSIGNMENT: Before class, review methods of determining concentrations of glucose, total protein, urea nitrogen, and chloride in body fluids.

STUDENT UNIFORM AND EQUIPMENT: Slide rule and laboratory notebook

TOOLS, EQUIPMENT, AND MATERIALS: Specimens and reagents for the determination of glucose, total protein, urea nitrogen, and chloride concentrations in body fluids, issued at the beginning of the first hour.

PERSONNEL: Three instructors (MOS 92B4H) 1st thru 8th hours. One instructor (MOS 92B4H) 9th hour.

INSTRUCTIONAL AIDS: Chalk, chalkboard, and eraser

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: GPE (1st thru 8th hours), C (9th hour)

FIRST THROUGH EIGHTH HOURS

I. INTRODUCTION (5 min)

A. Opening Statement: In clinical chemistry, the mastery of performing basic procedures is of utmost importance. A medical laboratory specialist may have vast knowledge of the chemical reactions occurring during the test procedures; however, his inability to satisfactorily perform routine determinations may well affect the patient's welfare.

B. Objectives

1. Satisfactorily perform determinations of glucose, total protein, urea nitrogen, and chloride concentrations in body fluids using methods learned during previous exercises.

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*This lesson plan supersedes LP 08-280-235, 081 and LP 08-280-285, 081.

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2. Accurately calculate and report results of routine determinations without supervision.

C. Class Procedure and Lesson Tie-in

Relates to hours previously presented on the determination of body fluid constituents.

II. APPLICATION (355 min)

A. Instructors' Activities

1. Provide specimens and reagents for the determination of glucose, total protein, urea nitrogen, and chloride concentrations in body fluids.

2. Supervise students' activities.

3. Collect and grade students' report sheets.

B. Students' Activities

1. Satisfactorily perform determinations of glucose, total protein, urea nitrogen, and chloride concentrations in specimens presented by the prescribed methods.

2. Maintain proper records.

    a. Laboratory notebook

    b. Quality control chart

3. Accurately calculate test results.

4. Report test values to instructors.

5. Clean area and equipment as necessary.

NINTH HOUR

III. CRITIQUE (45 min)

A. Questions from Students

B. Review of Main Points

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1. Errors in technique

2. Discrepancies in calculating and recording test results

3. Safety precautions

C. Closing Statement: The ability of a medical laboratory specialist to satisfactorily perform basic procedures is of utmost importance to the physician in treating his patients. The physician often relies on laboratory determinations as an aid to diagnosis of the patient's condition. As medical laboratory specialists, it behooves you to do your best to provide him with rapid and accurate test results.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-245-1
LABORATORY SCIENCES DIVISION 034
CHEMISTRY BRANCH

Acid-Base Equivalence in Body Compartmental Fluids

PLASMA**				INTERSTITIAL**				INTRACELLULAR**			
CATIONS 154 mEq/l.		ANIONS 154 mEq/l.		CATIONS 154 mEq/l.		ANIONS 154 mEq/l.		CATIONS 205 mEq/l.		ANIONS 205 mEq/l.	
Ion	Conc.≠	Ion	Conc.	Ion	Conc.	Ion	Conc.	Ion	Conc.	Ion.	Conc
Na^+	142	Cl^-	103	Na^+	145	Cl^-	115	Na^+	10	Cl^-	2
K^+	4	HCO_3^-	27	K^+	4	HCO_3^-	30	K^+	160	HCO_3^-	8
Mg^{++}	3	$HPO_4^=$	2	Mg^{++}	2	$HPO_4^=$	2	Mg^{++}	35	$HPO_4^=$	140
Ca^{++}	5	$SO_4^=$	1	Ca^{++}	3	$SO_4^=$	1				
		Organic-Acids	5			Organic-Acids	5				
		$Protein^-$	16			$Protein^-$	1			$Protein^-$	55
$HHCO_3$		Concentration		$HHCO_3$		Concentration		$HHCO_3$		Concentration	
1.38		1.38		1.50		1.50		0.40		0.40	

**These are mean values.

≠all concentrations expressed in mEq/liter.

*This mimeo supersedes M 08-280-240-1, 102.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-250
LABORATORY SCIENCES DIVISION 074
CHEMISTRY BRANCH

pH and Bicarbonate (8 hours)

COURSES PRESENTED TO: 311-92B20, Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES:

Annino, J. S. Clinical Chemistry: Principles and Procedures. 3rd ed. Boston: Little, Brown and Company, 1964, pp 78-87.
Arnow, L. E. Introduction to Physiological and Pathological Chemistry. 8th ed. St. Louis: C. V. Mosby Company, 1972, pp 85-88, 351-353.
Christensen, H. N. Neutrality Control in Living Organisms. Philadelphia: W.B. Saunders Company, 1971 pp 72-146.
Davenport, H. W. The ABC of Acid-Balance Chemistry. 5th ed. Chicago: University of Chicago Press, 1969.

STUDY ASSIGNMENT: After class, study lecture notes and review M 08-280-250-1.

STUDENT UNIFORM AND EQUIPMENT: Slide rule and laboratory notebook

TOOLS, EQUIPMENT, AND MATERIALS: M 08-280-250-1, one per student issued during the second hour. M 08-280-250-2, issued one per student at the beginning of the first hour

PERSONNEL: 1st thru 3rd hours - one instructor (MOS 92B4H)
4th thru 8th hours - eight instructors (MOS 92B4H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, pointer, eraser, VT 351 (see ANNEX A), receivers, and T 08-280-250-1 (see ANNEX D).

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: L (1st & 2nd hours), D (3rd hour), PE (4th thru 8th hours)

FIRST AND SECOND HOURS

NOTE: Issue M 08-280-250-1, one per student. Students take lecture notes on this mimeo.

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*This lesson plan supersedes LP 08-280-210, 081, and LP 08-280-315, 081.

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LP 08-280-250
074

I. INTRODUCTION (2 min)

A. Opening Statement: Metabolism is the active process of converting fuel to energy in the human body. Our body uses principally carbohydrates as fuel and converts them to carbon dioxide, water, and energy. One of these products, carbon dioxide, is commonly measured by you, the laboratory specialist. Carbon dioxide is not just a waste product of cellular respiration. The nervous system's control of breathing is determined to a great extent by the concentration of dissolved carbon dioxide in the body.

B. Objectives

1. Define pH and explain the meaning of the term "hydrogen ion concentration."

2. State the pH ranges for acidic, basic, and neutral solutions and list three methods of measuring pH.

3. Define "buffer system" and describe the function of buffer systems in maintaining acid-base balance in the body.

4. Explain the principles of carbon dioxide transport to include the function of carbonic anhydrase, the chloride shift, the formation of carbaminohemoglobin, and the formation of bicarbonate.

5. Briefly describe the metabolic conditions, alkalosis and acidosis.

6. With practice, satisfactorily perform titrimetric carbon dioxide determinations.

C. Class Procedure and Lesson Tie-in

Two hour lecture on pH, buffers, body buffer systems, and laboratory methods for bicarbonate to be followed by demonstration and practical exercise in laboratory methods for bicarbonate determination.

II. EXPLANATION (82 min)

A. pH

1. Definition

pH is defined as the negative $\log_{10}$ of the hydrogen ion concentration - $[H^+]$. pH indicates the hydrogen ion concentration and is used in the clinical laboratory to measure acidity or alkalinity of solutions and body fluids. Mathematically pH is expressed as: $pH = -\log_{10}[H^+]$.

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QUESTION: What determines the acidity of a solution?

ANSWER: Its hydrogen ion concentration.

2. pH scale
   a. 0 to 14
   b. Acid (0 to 7)
   c. Base (7 to 14)
   d. Neutral (7)

B. Videotape (12 min)(see ANNEX A)

1. Introduction
   a. Determination of pH (9:26 min)
   b. Points to be observed
      (1) pH scale
      (2) Indicator papers
         (a) Litmus
         (b) pHydrion
      (3) Indicator solutions
      (4) pH meter
2. Showing of videotape 351
3. Discussion and questions

C. Importance of pH

1. In reactions and determinations
2. In body fluids
   a. Blood (7.35 to 7.45 is normal)
   b. Urine pH (5 to 8 is normal)

QUESTION: What is the pH of normal human blood?

ANSWER: The pH range for normal human blood is 7.35 to 7.45.

D. Buffers

NOTE: See ANNEX B for definitions of an acid and a base.

1. Definitions

   a. A weak acid and salt of that acid

   b. A weak base and salt of that base

NOTE: Emphasize weak acid and weak base.

QUESTION: What comprises a buffer solution?

ANSWER: A buffer system is a solution which contains a weak acid (or base) and a salt of that acid (or base). This solution acts as a chemical sponge to resist a change in pH by soaking up strong acids or bases and forming weak acids or salts.

2. Mechanism of buffer systems

   a. Buffer system

      (1) $HCO_3^- + H^+ \longrightarrow HHCO_3$

      (2) $HHCO_3 + OH^- \longrightarrow HCO_3^- + H_2O$

   b. Addition of H^+

   c. Addition of OH^-

3. Buffer systems of the body

   a. Bicarbonate/carbonic acid

      (1) Main system of extracellular fluid (plasma)

      (2) Alkali reserve of the body

      (3) Maintenance of acid base balance in blood

NOTE: The bicarbonate/carbonic acid buffer system is important because the kidneys regulate the amount of bicarbonate ion in the blood while the lungs regulate the carbonic acid content by removing carbon dioxide from the blood.

- b. Hemoglobin
- c. Phosphate
- d. Protein

E. Carbon Dioxide and Bicarbonate

1. Formation of carbon dioxide
    - a. Glucose in tissue cells
    - b. Glycolysis -- CO_2 formation

QUESTION: What are the reaction products of complete carbohydrate metabolism?

ANSWER: Carbon dioxide, water, and energy.

2. Transportation of carbon dioxide

NOTE: Develop ANNEX C on the chalkboard as carbon dioxide transport is discussed.

- a. CO_2 diffuses into the plasma
    - (1) Dissolved CO_2 (about 5 percent of diffused)
    - (2) $H_2O + CO_2 \longrightarrow H_2CO_3 \longrightarrow H^+ + HCO_3^-$
- b. CO_2 diffuses into the erythrocytes
    - (1) That which is not dissolved in the plasma (95%)
    - (2) Ten percent forms carbaminohemoglobin
    - (3) Remainder enters reaction

$$CO_2 + H_2O \xrightarrow[\text{anhydrase}]{\text{carbonic}} H_2CO_3 \longrightarrow H^+ + HCO_3^-$$

NOTE: It is not necessary for the students to memorize the percentiles and reaction equations verbatim.

(4) Chloride shift

(a) HCO_3^- builds up in the cells

(b) HCO_3^- diffuses into the plasma

(c) Cl^- diffuses into the cells

(d) Maintains electrical neutrality

NOTE: Most of the carbon dioxide formed in the body is transported as bicarbonate ion in the plasma. This is the alkali reserve of the body.

QUESTION: What is the alkali reserve of the body?

ANSWER: The bicarbonate ion found in the plasma.

3. Removal of carbon dioxide

a. Exhalation from lungs

b. All reactions reversible

4. Summary

NOTE: Show T 08-280-250-1. (See ANNEX D)

F. Abnormal Conditions

1. Alkalosis

a. pH greater than 7.45

b. Increased HCO_3^-

c. Decreased $HHCO_3$

2. Acidosis

a. pH less than 7.35

b. Decreased HCO_3^-

c. Increased $HHCO_3$

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B. Laboratory Methods

1. Manometric

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a. Natelson microgasometer

b. Harleco CO_2 apparatus

2. Colorimetric - AutoAnalyzer

3. Titrimetric

a. $HCl + HCO_3^- \longrightarrow H_2CO_3 \longrightarrow H_2O + CO_2$

b. Titration of remaining HCl with NaOH

c. Normal values

(1) Adults - 21 to 30 mEq/L

(2) Children - 4 to 5 mEq/L less than adults

NOTE: Issue M 08-280-250-1, one per student.

H. Questions from Students

III. SUMMARY (6 min)

A. Review of Main Points

1. pH
2. Videotape
3. Importance of pH
4. Buffers
5. Carbon dioxide and bicarbonate
6. Abnormal conditions
7. Laboratory methods

B. Closing Statement: The importance of carbon dioxide in maintaining a balanced system within the patient's body warrants your extreme care when performing and reporting carbon dioxide determinations to the physician.

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THIRD THROUGH EIGHTH HOURS

IV. DEMONSTRATION (20 min)

NOTE: Refer the students to M 08-280-250-1 to follow the demonstrational steps as detailed in the mimeo.

- A. Principle of Method
- B. Preparation of Reagents
- C. Procedural Notes
- D. Normal Values
- E. Clinical Significance
- F. Procedural Steps
- G. Calculations
- H. Questions from Students

V. APPLICATION (235 min)

- A. Instructors' Activities
  1. Issue reagents and specimens
  2. Supervise student activities
  3. Make on-the-spot corrections of students' technique
  4. Grade students' reports
- B. Students' Activities
  1. Perform titrimetric carbon dioxide determinations according to M 08-280-250-1
  2. Calculate test results
  3. Report test results to instructors
  4. Perform necessary clean-up

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VI. CRITIQUE (15 min)

A. Questions from Students

B. Review of Noted Errors

1. Technique

2. Calculations

C. Closing Statement: All laboratory determinations must be performed with care and accuracy. The serum bicarbonate determination requires good pipetting technique in order to produce the desired accuracy.

ANNEX A
INSTRUCTIONAL AIDS

Videotape

VT No 351, 9:26 minutes. Determination of pH.

SYNOPSIS: Various technics for performing pH are discussed and demonstrated, including litmus paper, pHydrion paper, liquid indicators, and electronic pH meters. Composite pH machine is calibrated.

OBJECTIVES: Student will be able, after a sufficient amount of practical exercise, to make pH determinations using pH paper and pH indicators. Students will be able, after a sufficient amount of practical exercise, to calibrate single scale pH meters.

NOTE: Used in part IIB2 during the first hour.

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Definitions

1. The Bronsted-Lowry Concept of Acids and Bases.

Working independently in 1923, J. N. Bronsted in Copenhagen and J. M. Lowry in Cambridge proposed that an acid be defined simply as any substance that can donate a proton to any other substance. A base, on the other hand, is any substance that can accept a proton from any other substance. In short, an acid is a proton donor; a base is a proton acceptor.

2. Relative Strengths of Acids and Bases.

The strength of an acid, according to the Bronsted concept, is measured by its tendency to donate a proton. A strong acid is simply one which has a strong tendency to donate a proton. Similarly, the strength of a base is measured by its tendency to accept a proton. A strong base is one which has a strong tendency to accept a proton.

NOTE: Used in part IID during the first hour.

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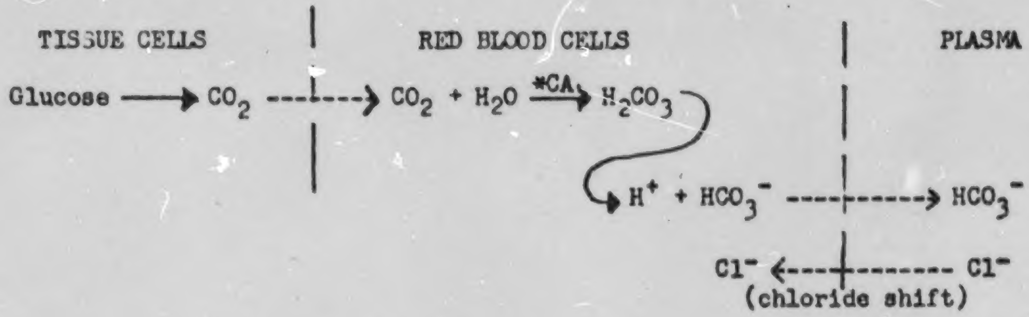
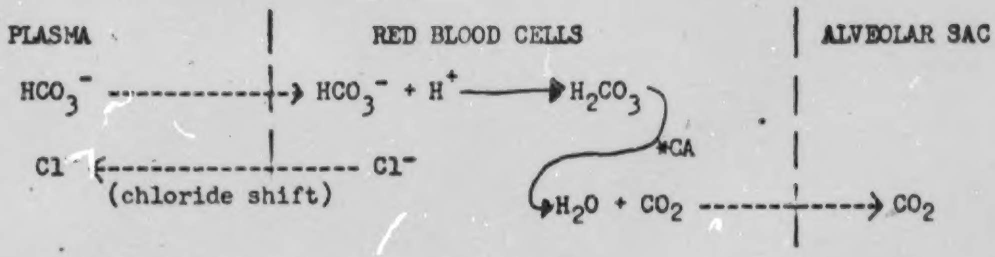
ANNEX C
INSTRUCTIONAL AIDS

Chalkboard Drawing

CELLULAR EXCHANGE


TISSUE CELLS
RED BLOOD CELLS
PLASMA
Glucose
CO2
CO2 + H2O
*CA
H2CO3
H+ + HCO3-
HCO3-
Cl-
Cl-
(chloride shift)

LUNG EXCHANGE


PLASMA
RED BLOOD CELLS
ALVEOLAR SAC
HCO3-
HCO3- + H+
H2CO3
*CA
Cl-
Cl-
(chloride shift)
H2O + CO2
CO2

*CA represents the enzyme carbonic anhydrase.

NOTE: Used in part IIE2 during the first hour.

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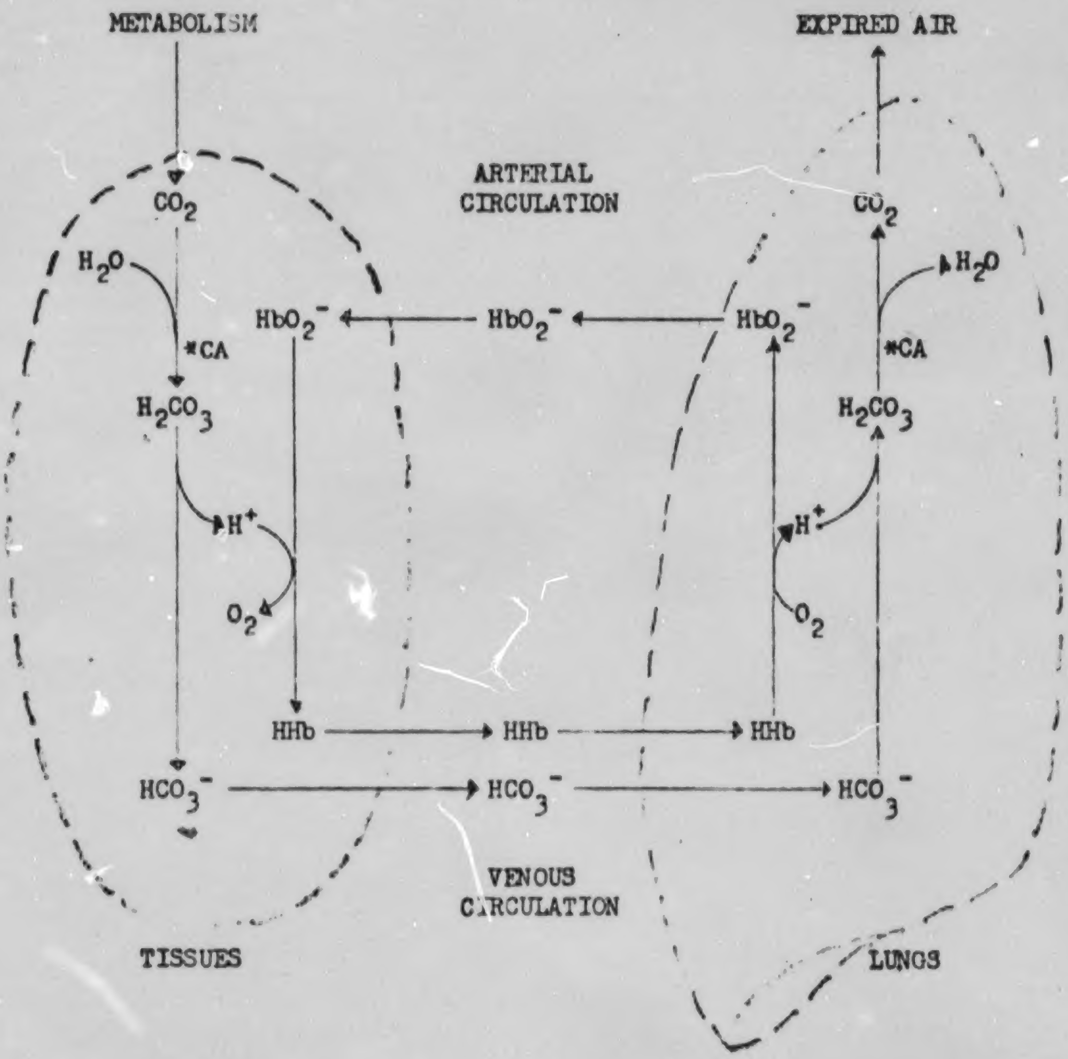
Transparency

T 08-280-250-1. (As diagramed below)

Review of CO_2 Transport


METABOLISM
EXPIRED AIR
ARTERIAL CIRCULATION
CO_2
H_2O
*CA
H_2CO_3
H^+
O_2
HbO_2^-
HbO_2^-
HbO_2^-
CO_2
H_2O
*CA
H_2CO_3
H^+
O_2
HHb
HHb
HHb
HCO_3^-
HCO_3^-
HCO_3^-
VENOUS CIRCULATION
TISSUES
LUNGS

*CA represents the presence of the enzyme carbonic anhydrase.

NOTE: Used in part IIE2 during the first hour.

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LABORATORY SCIENCES DIVISION 074
CHEMISTRY BRANCH

pH and Bicarbonate

Metabolism is the active process of converting fuel to energy in the human body. Our body uses principally carbohydrates as fuel and converts them to carbon dioxide, water, and energy. One of these products, carbon dioxide, is commonly measured by you, the laboratory specialist. Carbon dioxide is not just a waste product of cellular respiration. The nervous system's control of breathing is determined to a great extent by the concentration of dissolved carbon dioxide in the body.

To best understand the role of carbon dioxide in the body, we must first develop a working definition for pH. pH simply means the concentration of the hydrogen ions and is a way in which we can measure the acidity or alkalinity of a solution. Expressed mathematically, $\text{pH} = -\log_{10}[H^+]$. This means that instead of saying the concentration of the hydrogen ion is 0.00001 M, we can state that the pH is 5; i.e., $-\log(0.00001) = 5 = \text{pH}$.

The pH scale has a range from zero to fourteen. On this scale, any solution with a pH below seven is acidic, the stronger concentrations of acid being closer to zero on the scale. Alkaline solutions, therefore would be those that have a pH greater than seven on the scale, the stronger concentration of base being closer to fourteen. A pH of seven represents neutral solutions.

The following videotape is entitled Determination of pH. Some of the more important aspects of this tape are a brief discussion of the pH scale and various methods which may be used to measure the pH of a solution. Indicator papers and indicator solutions are mentioned briefly, but most of the time is devoted to the use of the pH meter.

pH is important because certain reactions and determinations require a specific pH range in order to go to completion. This means that different body fluids must maintain fairly narrow pH ranges. Human blood, for example, has a normal pH range of 7.35 to 7.45 (mean = 7.4). Urine, on the other hand, has a normal pH range of 5 to 8; showing fluctuations from day to day with changes in body needs.

In order to maintain these narrow pH ranges we need something that will neutralize the effects of acids and bases which are produced by the body. This is accomplished by what we call buffers. We may define a buffer as a solution that contains a weak acid (or a weak base) and the salt of that acid (or base).

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*This instructor's manuscript supersedes IM 08-280-210, 081 and IM 08-280-315, 081.

There are several buffer systems in the body, the most important of which is the carbonic acid-bicarbonate system. This system is the main buffer system of the extracellular fluid and is found in the plasma. Since the ratio of bicarbonate ion to carbonic acid is 20:1, we say that it is the alkali reserve of the body. This is an important buffer system because it can be regulated by both the kidneys (bicarbonate ion) and the lungs (carbonic acid). Some of the other buffers in the body are hemoglobin, phosphate, and protein.

The carbon dioxide, which is used in this carbonic acid-bicarbonate ion buffer system, is formed when glucose at the cellular level undergoes glycolysis. This glycolysis releases carbon dioxide, water, and energy. The carbon dioxide, which is essentially a waste product of carbohydrate metabolism, is transported from the cells to the lungs in three forms. First, the carbon dioxide diffuses from the tissue cells to the plasma where a small amount (about five percent) is transported as dissolved CO_2. This dissolved carbon dioxide will to some extent enter into the reaction

$$H_2O + CO_2 \longrightarrow H_2CO_3 \longrightarrow H^+ + HCO_3^-$$

(This reaction is very slow at pH 7.4)

but most of it will remain physically dissolved. The remaining carbon dioxide (about ninety-five percent) will diffuse into the erythrocytes. Some of this diffused carbon dioxide (approximately 10%) will react with hemoglobin to form carbaminohemoglobin while the rest, in the presence of the enzyme carbonic anhydrase, will speed through the reaction

$$H_2O + CO_2 \xrightarrow[\text{anhydrase}]{\text{carbonic}} H_2CO_3 \longrightarrow H^+ + HCO_3^-$$

As can be seen from the above equation, there is a rapid buildup of the bicarbonate ion inside the cell. These bicarbonate ions diffuse from the erythrocytes into the plasma and are replaced in the erythrocytes by chloride ions from the plasma. This movement of chloride ions into the erythrocytes and the bicarbonate ions into the plasma is called the chloride shift. As bicarbonate moves out of the cell, there is a buildup of positive charge inside the RBC; to maintain electrical neutrality Cl^- diffuses into the cell.

When the erythrocytes reach the capillary beds of the lungs, all the reactions that we have discussed will take place in reverse. The carbon dioxide gas will diffuse from the blood to the alveolar sac and then be exhaled from the body.

There are certain abnormal conditions which result when there is an upset in the ratio of bicarbonate ions to carbonic acid. In the condition known as alkalosis, there is usually an increase in the amount of bicarbonate ions present or a decrease in the amount of carbonic acid that is present. This will cause the pH of the blood to be greater than 7.45; hence the name alkalosis, for we are on the basic end of the blood's normal pH range. Acidosis, on the other hand, is a condition resulting from a decrease in the bicarbonate ion or an increase in the carbonic acid concentrations. In this case, the pH will be less than 7.35. Although the overall pH of the blood is alkaline, it is lower than the normal blood pH range and we therefore call this condition acidosis.

There are several methods for determining carbon dioxide concentrations in body fluids. A manometric procedure utilizing the Natelson microgasometer or the Harleco Carbon Dioxide apparatus, and an AutoAnalyzer method are commonly used in the laboratory. For our work in this class, we will perform yet another method known as a titrimetric carbon dioxide determination. In this method, we actually measure the concentration of bicarbonate ion in the specimen rather than the total carbon dioxide content.

The titrimetric carbon dioxide method is performed by mixing a known amount of hydrochloric acid with the serum sample. We then swirl the container a set period of time to facilitate the release of gaseous carbon dioxide from the bicarbonate ions in the sample. By titrating the remaining hydrochloric acid (that which has not reacted with the bicarbonate ions) with sodium hydroxide, we can determine the amount of hydrochloric acid that has been used in the reaction and with this information, we can calculate the concentration of carbon dioxide that was released in the reaction. Normal values for the titrimetric carbon dioxide method range from twenty-one to thirty (21 to 30) mEq/L of serum for adults and are from four to five (4 to 5) mEq/L lower than the adult values for children.

During this period of instruction, we have discussed pH and have viewed a videotape on the determination of pH. We also discussed the importance of pH and the important roles of buffer systems in the body in maintaining pH. We discussed the formation, transport, and removal of carbon dioxide from the body, and abnormal conditions which may result when body carbon dioxide concentrations change. We also discussed methods for measuring carbon dioxide concentrations in body fluids.

The importance of carbon dioxide in maintaining a balanced system within the body warrants your extreme care when performing and reporting carbon dioxide determinations to the physician.

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SP5 Gary L. Wilcox

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-250-1
LABORATORY SCIENCES DIVISION 037
CHEMISTRY BRANCH

Harleco Micro CO_2

A. Principle

A known amount of liberating solution (lactic acid) is added to a sample (serum) which is agitated at a controlled rate and releases carbon dioxide as a gas. The gas released moves a measured amount of indicator fluid up the gasometric capillary tube proportional to the carbon dioxide content of the sample. Comparison of the unknown sample to a standard of known concentration allows for easy calculation.

B. Notes and Precautions

1. The Harleco Micro CO_2 system represents a simplified version of the Van Slyke method. This system provides a means for the determination of total CO_2 content in serum in approximately one minute.

2. For routine clinical analysis clotted venous blood collected anaerobically may be used. The specimen should be placed in a stoppered container and the test performed as soon as possible after collection of the blood. Separation of the serum from the erythrocytes should be carried out immediately to prevent metabolism in the erythrocytes from altering the bicarbonate concentration. If plasma is used for CO_2 determinations, tripotassium EDTA, sodium fluoride with potassium oxalate, and sodium oxalate falsely depress CO_2 values. Lipemic serum also depresses the CO_2 values, because it increases the solubility of CO_2.

3. Atmospheric pressure and temperature will vary readings for the Harleco Micro CO_2 procedure. Therefore, it is important to rerun the standard for each set of controls and unknowns.

C. Reagents

1. Stock lactic acid 85%

2. Micro CO_2 liberating solution (lactic acid 21.25%)

   Place approximately 500 ml of deionized water into a liter volumetric flask and slowly, with constant swirling, add 250 ml of the lactic acid 85%. QS to the mark with deionized water and thoroughly mix by inversion.

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*This mimeo supersedes M 08-280-250-1, 074.

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3. Micro CO_2 gasometric indicator fluid

   Place .01 g of methylene blue into a 100 ml volumetric flask. Add approximately 0.5 ml of a water based surfactant (such as Brij 35) to flask and qs with water to mark. Mix thoroughly by inversion and dissolve the methylene blue completely.

4. CO_2 working standard (25 mEq/L)

   Place 2.1 g ACS grade sodium bicarbonate ($NaHCO_3$) into a one liter volumetric flask. QS to mark with deionized water and mix thoroughly by inversion.

D. Specimen of Choice

Serum

E. Equipment Needed

1. 64989 Micro CO_2 apparatus
2. Micro CO_2 liberating solution
3. Micro CO_2 gasometric indicator fluid
4. Gasometric capillary tubes
5. Urethane gasket - rectangular
6. Silicone gasket - round
7. Reaction vessels (plastic cups)
8. 10 ml serological pipets
9. 100 microliter Eppendorf pipet with disposable tips

F. Procedural Steps

1. Inspect reaction vessels carefully for hairline cracks, especially the upper rims. Vessels should have been rinsed well in tap water (3 - 5 times) and once in deionized water.

2. Using the 10 ml serological pipet with line-to-line delivery, pipet 0.5 ml of liberating solution (lactic acid) into one side of each of the reacting vessels, avoiding any spillage into empty side.

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NOTE: Discard Kimwipe^{R} used to wipe pipet (if reused it will cause erroneous results in step 3).

3. Using a 100 microliter Eppendorf pipet, pipet 0.1 ml of unknown, standard, and control into empty sides of appropriately labeled vessels, using a different pipet for each.

Sample Preparation

	Liberating Solution	Standard	Control	Unknown
Standard	0.5 ml	0.1 ml	-------	-------
Control	0.5 ml	--------	0.1 ml	-------
Unknown #1	0.5 ml	--------	-------	0.1 ml
Unknown #2	0.5 ml	--------	-------	0.1 ml

4. Check the Micro CO_2 apparatus by placing an empty reaction vessel into the well and tightening thumb screw until the top of the fluid reaches the zero mark. The fluid should rise slowly above zero. If it stays motionless or falls and tightening thumb screw will not solve problem, check the reaction vessel to see if it is cracked; if not, the seal and gasket may need to be replaced (call an instructor).

5. Check indicator fluid in capillary tube - it should be about 1 cm long.

   a. If there is not enough indicator fluid in capillary tube carefully remove the capillary tube from the 64989 Micro CO_2 Apparatus using capillary action, fill the capillary with the correct amount.

   b. If there is too much indicator fluid in capillary tube carefully remove the capillary tube from the 64989 Micro CO_2 Apparatus. Using a Kimwipe, remove excess amount of indicator fluid.

6. Place the standard cup into the well and tighten the thumb screw until the top of the indicator fluid is at the zero mark.

7. Wait at least 20 seconds.

8. Use thumb screw to readjust fluid level to zero mark.

9. Press start button.

10. When machine shuts off, wait approximately 1 second for fluid level to stabilize, then read and record top level of fluid.

11. Place finger over top of capillary tube, release thumb screw and open hinge.

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12. Remove cup and carefully wipe gasket dry.

NOTE: Even trace amounts of moisture left on gasket will affect next reading.

13. Repeat steps 6 - 12 for the control and unknowns.

G. Calculations

1. The CO_2 content of serum is calculated from the following:

   a. $$\frac{\text{Concentration of Standard}}{\text{Reading of Standard}} = \text{K Factor}$$

   b. K Factor x Reading of Unknowns = Concentration of Unknown

2. Example:

   If standard reading is 26
   Unknown #1 reading is 21
   Unknown #2 reading is 28

   a. $$\frac{\text{Concentration of Standard}}{\text{Reading of Standard}} = \text{K Factor}$$

   $$\frac{25}{26} = \text{K Factor}$$

   $$.96 = \text{K Factor}$$

   b. K Factor x Reading of Unknown or Control = Concentration of Unknown

   .96 x 21 (Unknown #1 reading) = 20 mEq/L

   .96 x 28 (Unknown #2 reading) = 27 mEq/L

H. Normal Values

Adults: 21 to 30 mEq/L
Children: Values may be 4 to 5 mEq/L lower than adult values

I. Clinical Significance

1. Abnormal values indicate electrolyte imbalance.

2. Abnormal values are found in respiratory and metabolic malfunctions.

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3. References

Reiner, M., Standard Methods of Clinical Chemistry. New York: Academic Press Incorporated, 1953, pp. 19-22.

Henry, R. J., Cannon, D. C., and Winkelman, J. W., Clinical Chemistry Principles and Techniques. New York: Harper and Row, 1974, pp. 784.

Harleco, The Micro CO_2 System. Gibbstown, N.J.: Harleco, Division of American Hospital Supply Corporation, 1975.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-255
LABORATORY SCIENCES DIVISION 068
CHEMISTRY BRANCH

Sodium and Potassium (10 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES:

TM 8-227-6, Chap 11.
Bland, J. H. Clinical Metabolism of Body Water and Electrolytes. Philadelphia: W. B. Saunders Company, pp 3-40, 1963.
Tietz, N. Fundamentals of Clinical Chemistry. 2nd ed. Philadelphia: W. B. Saunders Company, pp 873-879, 1976.
Davidsohn and Henry. Clinical Diagnosis by Laboratory Methods. Philadelphia: W. B. Saunders Company, pp 773-776, 1974.
Lee, L. W. Elementary Principles of Laboratory Instruments. 3rd ed. St. Louis: C. V. Mosby Company, 1974, Chap 5.
Operators Manual 143 Flame Photometer. Lexington: Instrumentation Laboratory Incorporated, 1969.

STUDY ASSIGNMENT: After class, review lecture notes and M 08-280-255-1.

STUDENT UNIFORM AND EQUIPMENT: Laboratory notebook

TOOLS, EQUIPMENT AND MATERIALS: M 08-280-255-1, issued one per student at the beginning of class.

PERSONNEL: One instructor (MOS 92B4H), 1st hour and 8th hour. Eight instructors (MOS 92B4H), 2nd thru 7th hours and 9th and 10th hours.

INSTRUCTIONAL AIDS: Chalk, chalkboard, eraser; reagents; IL 143 Flame Photometer.

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture (1st hour); PE (2nd thru 7th hours and 9th and 10th hours); Conference (8th hour).

FIRST HOUR

I. INTRODUCTION (5 min)

A. Opening Statement: A patient who has lost a considerable

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*This lesson plan supersedes LP 08-280-255, 084.

amount of body fluid because of a traumatic injury, severe burns, vomiting, or other clinical conditions also will have lost the electrolytes that the fluid contained. In replacing the fluid and restoring electrolyte balance, the physician must have current electrolyte values on the patient. Two key electrolytes are sodium and potassium; their concentrations are measured by a flame emission photometer.

B. Objectives

1. Select the principle of flame photometry.

2. Select the normal ranges for serum sodium and potassium.

3. Select the chief cation in intracellular and in extracellular fluids.

4. With practice, satisfactorily perform sodium and potassium determinations.

NOTE: Issue M 08-255-1, one per student.

C. Class Procedure and Lesson Tie-in: A practical exercise on the operation of the IL 143 Flame Photometer to measure sodium and potassium will follow this lecture.

II. EXPLANATION (35 min)

A. Sodium (Natrium)

1. Physiology

a. Chief extracellular cation

b. Function

(1) Activates certain enzymes

(2) Promotes nerve and muscle irritability (response to stimulus)

(3) Significant in H_2O reabsorption

c. Absorbed in the small intestine

d. Blood concentration regulated by the kidney

2. Clinical significance

a. Normal values

136-145 mEq/L

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- b. Increased (hypernatremia)
  - (1) Dehydration
  - (2) Renal failure
- c. Decreased (hyponatremia)
  - (1) Polyuria - diabetes insipidus
  - (2) Diarrhea

QUESTION: Sodium is the chief cation in which body compartment?

ANSWER: Sodium is the chief extracellular cation.

F. Potassium (kalium)

1. Physiology
   - a. Chief intracellular cation
   - b. Function
     - (1) Promotes nerve and muscle irritability
     - (2) Helps maintain heart rhythm
   - c. Absorbed in the small intestine
   - d. Blood concentration regulated by the kidney
2. Clinical significance
   - a. Normal value

     3.5-5.0 mEq/L
   - b. Increased (hyperkalemia)
     - (1) Renal failure
     - (2) Renal obstruction
   - c. Decreased (hypokalemia)
     - (1) Diarrhea
     - (2) Vomiting

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C. Laboratory Methods

   1. Colorimetric (historic)

   2. Ion specific electrode

   3. Flame photometry

D. Flame Photometry

   1. Principle - emission spectroscopy

   2. Internal operation

      a. Metal ions are atomized and burned in a pure flame

      b. Metal ions emit light of a characteristic wavelength when burned

      c. Intensity of light emitted is proportional to concentration

      d. Lithium used as an internal standard

   3. Procedural precautions

      a. Sodium and potassium salts contained in some anticoagulants elevate values

      b. Serum should be non-hemolyzed

QUESTION: What is the principle of flame photometry?

ANSWER: Emission spectroscopy is the principle of flame photometry.

E. Questions From Students

III. SUMMARY (5 min)

A. Review of Main Points

   1. Sodium

   2. Potassium

   3. Laboratory methods

   4. Flame Photometry

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B. Closing Statement: The Army has kept pace in developing a rapid, efficient treatment for emergency cases. Since many emergency cases involve fluid loss and the upset of electrolyte balance, "STAT" sodium and potassium determinations are an important part of this treatment. Proper use of the flame photometer will allow you to supply accurate results of these determinations.

SECOND THROUGH SEVENTH HOURS AND NINTH AND TENTH HOURS

IV. APPLICATION (350 min)

A. Instructors' Activities

1. Step by step, orient the students to the preparation of reagents and samples for sodium and potassium determinations; the warm-up, standardization, use, and shutdown of the IL 143 Flame Photometer; errors caused by improper dilutions; improperly adjusted aspirator; hemolyzed sera; and the effects of test contamination such as perspiration, detergent residue in the glassware, dirty glassware, and fingerprints in the sample cups.

2. Provide reagents and samples

3. Supervise student activities

4. Make on-the-spot corrections of errors in student technique

5. Collect and grade student test reports

B. Students' Activities

1. Become oriented to procedures for the use of the IL 143 Flame Photometer

2. Prepare sample and standard dilutions

3. Perform sodium and potassium determinations using the IL 143 Flame Photometer to within ± 5 percent for sodium and ± 10 percent for potassium.

4. Check coefficient of variation with instructors

5. Submit test reports to the instructors

6. Clean up area and equipment as necessary

V. REVIEW (10 min)

A. Questions from Students

B. Review Student Errors

1. Technique

2. Safety

C. Precautions

1. Contamination of test

2. Safety

D. Closing Statement: The Flame Photometer is a simple instrument to operate and, when used properly, will give accurate test results.

EIGHTH HOUR

VI. CONFERENCE (45 min)

A. Directions to Students

1. Put away all lecture notes and texts

2. M 08-280-205-1 may be used

B. Instructor's Activities

1. Issue quiz sheet, one per student

2. Supervise students' activities

3. Clarify any vague points

4. Collect completed quiz sheets

5. Grade completed quiz sheets

C. Students' Activities

1. Place identification on quiz sheet

2. Answer all questions as appropriate

3. Turn completed quiz sheet in to the instructor for grading

D. Critique

1. Questions from students

2. Reteach instructional material as necessary

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *IM 08-280-255
LABORATORY SCIENCES DIVISION 068
CHEMISTRY BRANCH

Flame Photometer

A patient who has lost a considerable amount of body fluid because of a traumatic injury, severe burns, vomiting, or other clinical conditions will also have lost the electrolytes that the fluid contained. In replacing the fluid and restoring electrolyte balance, the physician must have current electrolyte values on the patient. Two key electrolytes are sodium and potassium; their concentration measurement is by a flame emission photometer.

Sodium (natrium) is the major cation of extracellular fluid. It has many important functions in the body. It plays a central role in the maintenance of normal distribution of water in the various fluid compartments; and it activates certain enzymes and promotes nerve and muscle irritability. Most of the sodium which is taken into the body is absorbed in the small intestines. The overall body concentration of sodium is regulated by the kidney.

A decrease or increase of sodium in various body fluids is clinically significant. The normal range for serum sodium is 136-145 mEq/L. A significant increase of sodium above that range creates a condition known as hypernatremia. Dehydration and renal failure can cause hypernatremia. A significant decrease in serum sodium is called hyponatremia. Polyuria (diabetes insipidus) and diarrhea can cause this condition.

Potassium (kalium) is the chief intracellular cation. Its functions are to promote nerve and muscle irritability and to assist in maintaining heart rhythm. Potassium is absorbed in the small intestines and the blood concentration is regulated by the kidney.

A decrease or increase in serum potassium is clinically significant. The normal range is 3.5-5.0 mEq/L. A significant increase of potassium is called hyperkalemia; renal failure and renal obstruction can cause this condition. A decrease in serum potassium (hypokalemia) can be caused by diarrhea and vomiting.

Laboratory methods for determining sodium and potassium utilize ion specific electrodes or flame photometry. Flame photometry is the method of choice. Its principle is emission spectroscopy. The internal operation of a flame photometer has the following pattern: metal ions are atomized and burned in a pure flame; metal ions emit light of a characteristic wavelength when burned; intensity of light emitted is proportional to concentration; lithium is used as an internal standard.

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*This instructor's manuscript supersedes IM 08-280-255, 084.

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When performing a sodium and potassium determination, one should be aware of certain procedural precautions. Na^+ and K^+ salts are contained in some anticoagulants which could elevate values erroneously. These anticoagulants should be avoided during specimen collection. Hemolyzed serum can also give false results; it causes the potassium to be falsely elevated. Proper technique during specimen collection can eliminate this problem.

Today we have covered sodium, potassium, laboratory methods, and flame photometry. The Army has kept pace in developing a rapid, efficient treatment for emergency cases. Since many emergency cases involve fluid loss and the upset of electrolyte balance, "STAT" sodium and potassium determinations are an important part of this treatment. Proper use of the flame photometer will allow you to supply these determinations.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-255-1
LABORATORY SCIENCES DIVISION 083
CHEMISTRY BRANCH

Sodium and Potassium
Flame Emission Photometry: The IL 143

A. PRINCIPLE

Flame emission photometry measures the intensity of emitted light produced by a burning flame at characteristic wavelengths. If a solution containing a particular metal ion (sodium or potassium) is introduced into the flame, the metal ions are subjected to a source of energy and the electrons in the outermost orbit around the nucleus are raised to a higher energy level. As the electrons return to their former ground state, energy is given off in the form of light of a specific wavelength. Increases in this light's intensity are directly proportional to the concentration of the particular metal in the flame.

B. REAGENTS

1. Lithium diluent, 15 mEq/L. Make a 1:100 dilution of the stock lithium solution using deionized water as the diluent.

2. Stock standard solution, 140 mEq sodium/L and 5.0 mEq potassium/L. Available from Instrumentation Laboratory, Incorporated.

C. PROCEDURAL NOTES

1. When making the working standard solution, double volumes of the solutions may be used (40 microliters of stock standard solution and 8.0 ml of lithium diluent). The larger total volume insures that there is adequate solution to standardize the instrument.

2. If the lithium response meter is outside the two lines at anytime during your determinations, correct it with the lithium set control, then rezero and restandardize the instrument before continuing analysis. The instrument must be rezeroed and restandardized any time the lithium set control is moved.

3. Standards, samples, and blanks can be easily contaminated by dirty or soapy glassware or perspiration from fingers touching the inside of the glassware or the tips of the pipets.

4. When immersing the aspirating needle into the sample cups, be careful not to bump the needle. The needle may be bent or pushed upward enough to alter or stop the rate of aspiration.

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*This mimeo supersedes M 08-280-310-1, 092.

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D. NORMAL VALUES

1. Serum sodium - 138 to 146 mEq/L.

2. Serum potassium - 3.5 to 5.0 mEq/L.

E. PROCEDURE

1. IL 143 components

a. Front panel - see page 4.

b. Rear panel - see page 5.

2. Sample preparation.

a. Make the following dilutions in appropriately marked Disposo-Cups.

	BLANK "B"	STANDARD "S"	CONTROL "C"	UNKNOWNS "U-1", etc
Lithium diluent solution	4.0 ml	4.0 ml	4.0 ml	4.0 ml
Stock standard solution	- - -	20 µl	- - -	- - -
Control serum	- - -	- - -	20 µl	- - -
Unknown serum	- - -	- - -	- - -	20 µl

b. If these dilutions are not used immediately, cover the cups with parafilm.

3. IL 143 operating procedure. (NOTE: The numbers in parentheses refer to the schematic diagrams of the IL 143 which are found on pages 4 and 5.)

a. Ignition.

(1) Turn the propane Screw Valve (17) completely OPEN.

(2) After 10 seconds, turn the On/Off Switch (13) to ON. If the Flame On Indicator Light (10) does not come ON within a few seconds, turn OFF the instrument and inform the instructor immediately.

b. Warm up the instrument for 5 minutes while aspirating lithium diluent.

c. Zero the instrument.

(1) Aspirate the BLANK solution.

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(2) Adjust the Lithium Response Meter (11) to the center of the reference triangle using the Lithium Set Control (12).

(3) Using the Sodium (3) and Potassium (7) Zero Control knobs, set the Sodium (1) and Potassium (5) Digital Displays to zero.

d. Standardize the instrument.

(1) Aspirate the dilute STANDARD solution.

(2) Check the Lithium Response Meter (11).

(3) Using the Sodium (2) and Potassium (6) Balance Control knobs, set the Sodium Digital Display (1) to 140 and the Potassium Digital Display (5) to 5.0.

(4) Rerun the zeroing and standardizing procedures several times and make any necessary adjustments.

e. Quantitate the samples.

(1) Aspirate the CONTROL and UNKNOWN dilutions in turn.

(2) With each aspiration, check the Lithium Response Meter (11).

(3) With each aspiration, record the values from the Sodium (1) and Potassium (5) Digital Displays.

f. Shut down the instrument.

(1) Aspirate a fresh BLANK solution for 5 minutes.

(2) Aspirate deionized water for 5 minutes.

(3) Turn the propane Screw Valve (17) completely CLOSED.

(4) The No Gas Indicator Light (8) will come ON.

(5) The Flame On Indicator Light (10) will go OUT.

(6) After the Flame On Indicator Light (10) goes out, turn the On/Off Switch (13) to OFF.

F. REFERENCE

Instructions, IL 143 Digital Flame Photometer. #471/79143. Lexington: Instrumentation Laboratory Incorporated, 1969.

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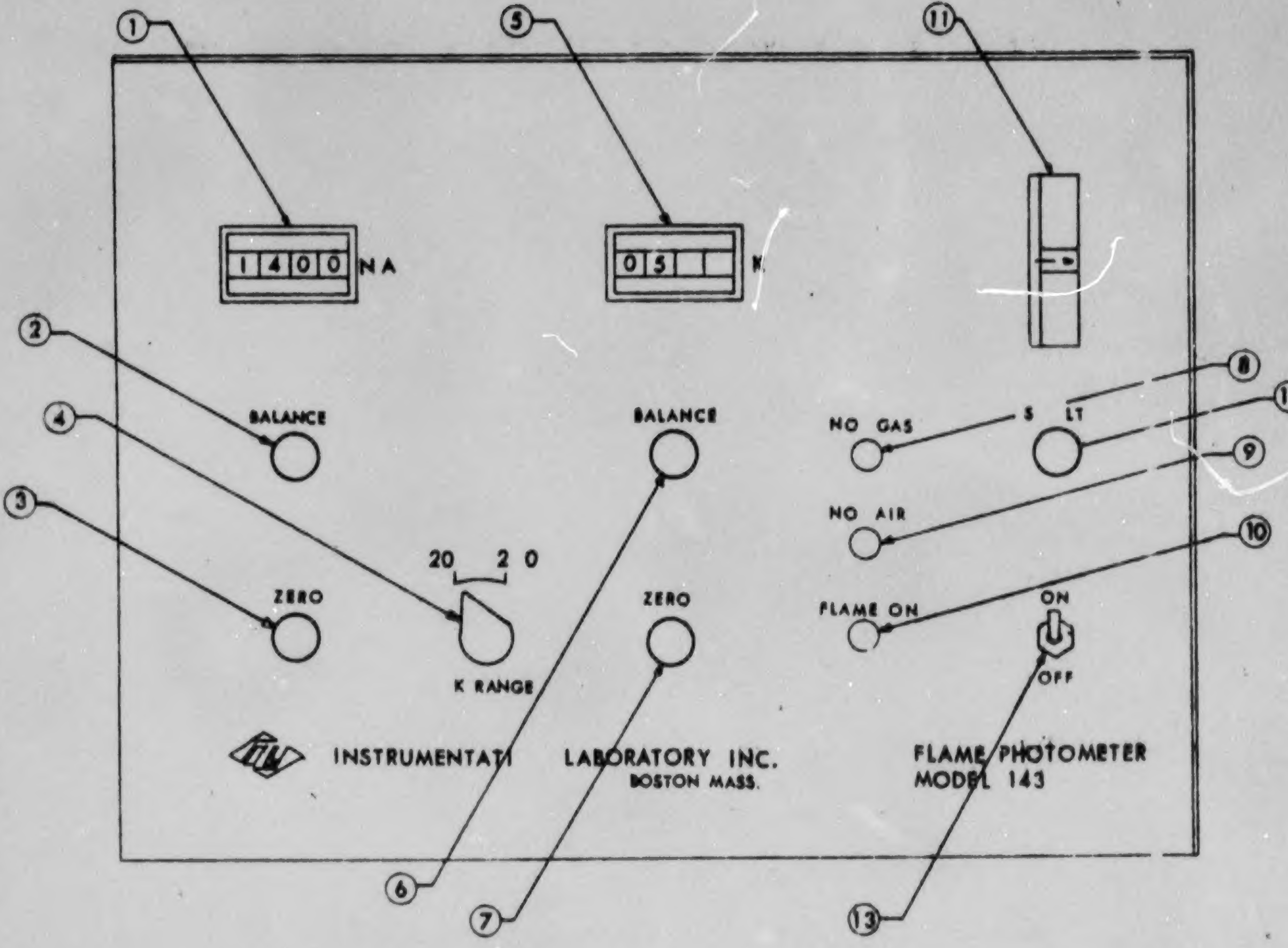
FRONT PANEL:



①
⑤
⑪
1400 NA
05 K
②
BALANCE
④
③
ZERO
20 2 0
K RANGE
BALANCE
ZERO
NO GAS
S LT
⑧
⑫
⑨
NO AIR
⑩
FLAME ON
ON
OFF
INSTRUMENTATI LABORATORY INC.
BOSTON MASS.
FLAME PHOTOMETER
MODEL 143
⑥
⑦
⑬

1. Sodium digital concentration display
2. Sodium balance control
3. Sodium zero control
4. Potassium range selector switch
5. Potassium digital concentration display
6. Potassium balance control
7. Potassium zero control
8. No gas indicator light
9. No air indicator light
10. Flame on indicator light
11. Lithium response meter
12. Lithium set control
13. On/off switch

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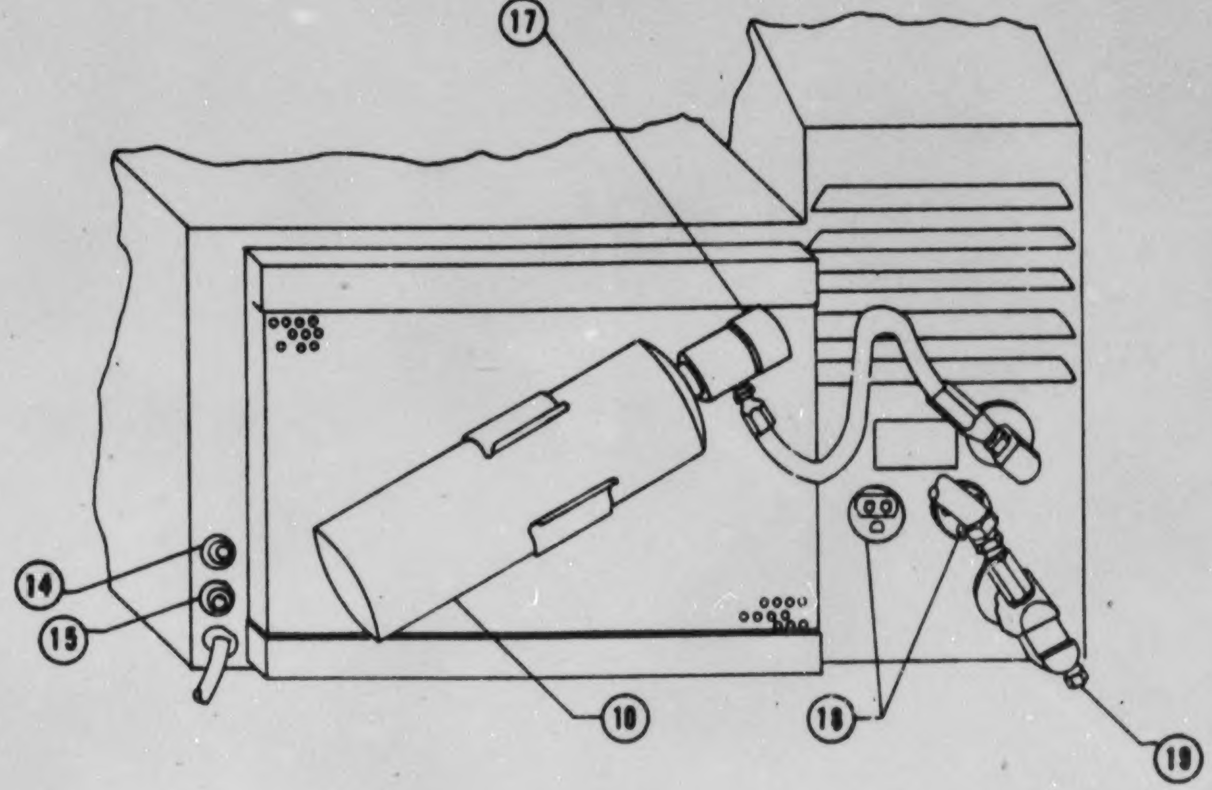
14. REAR PANEL:



14. Instrument fuse
15. Accessory fuse
16. Propane fuel cylinder
17. Screw valve
18. Accessory outlets
19. Moisture bleed fitting

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-260
LABORATORY SCIENCES DIVISION 084
CHEMISTRY BRANCH

Bilirubin (9 hours)

COURSES PRESENTED TO: 311-92B20, Medical Laboratory Procedures (Basic)

PLACE: Classroom/laboratory

REFERENCES:

Arnow, L. E. Introduction to Physiological and Pathological Chemistry. 8th ed. St. Louis: C. V. Mosby Company, 1972, chap 25.
Guyton, A. C. Textbook of Medical Physiology. 4th ed. Philadelphia: W. B. Saunders Company, pp 867-868, 1971.
Lucey, J., Ferreiro, M., and Hewitt, J. "Prevention of Hyperbilirubinemia by Phototherapy." Pediatrics, 41, 1047-1054, 1968.

STUDY ASSIGNMENT: After class, review lecture notes and M 08-280-260-1

STUDENT UNIFORM AND EQUIPMENT: Slide rule and laboratory notebook

TOOLS, EQUIPMENT, AND MATERIALS: M 08-280-260-1 issued one per student at the end of the first hour. M 08-280-260-2 issued one per student at the beginning of the first hour. Reagents and specimens for the Evelyn-Malloy serum bilirubin determination issued to the students at the beginning of the second hour.

PERSONNEL: 1st and 9th hours, one instructor (MOS 92B4H); 2d thru 8th hours, eight instructors (MOS 92B4H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, eraser, overhead projector, and T 08-280-260-1 thru -5 (see ANNEX A)

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: L (1st hour), PE (2d thru 8th hours), EX (9th hour)

FIRST HOUR

NOTE: Issue M 08-280-260-2, one per student. Students take lecture notes on this mimeo.

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*This lesson plan supersedes LP 08-280-295, 081.

I. INTRODUCTION (5 min)

A. Opening Statement: One of the most important organs of the body is the liver which plays a role in a vast number of the body's processes. Therefore, it is important that some means of measuring the efficiency of liver functions be available. One test for determining some of the liver's functions is based on quantitating a bile pigment called bilirubin.

B. Objectives

1. List and discuss the three basic functions of the liver.
2. Define "bilirubin" and "reticuloendothelial system (R.E.S.)."
3. List the two basic forms of bilirubin, their common synonyms, and state the normal serum values of each.
4. List and discuss the four types of jaundice, including a related cause and type of bilirubin that is increased due to each.
5. Outline the formation and excretion of bilirubin in the body.
6. With practice satisfactorily perform the Evelyn-Malloy bilirubin determination.

C. Class Procedure and Lesson Tie-in

1. First of nine hours on bilirubin
2. Practical exercise and examination to follow

II. EXPLANATION (35 min)

A. Functions of the Liver

1. Vascular
    a. Blood flow
        (1) Portal vein - 1000 ml/min
        (2) Hepatic artery - 400 ml/min
    b. Filtration

(1) Kupffer cells

(2) Phagocytization of bacteria

c. Detoxification

(1) Metabolize toxins

QUESTION: What type of compounds ingested are considered to be toxins?

ANSWER: Alcohol and drugs. Ammonia, a normal metabolic waste product, is also toxic to the body.

(2) Conjugate products - formation of glucuronides

(3) Slow process

2. Metabolic

a. Carbohydrates

(1) Glycogen storage

(2) Glycolysis

(3) Gluconeogenesis

b. Lipids

(1) Fat interconversions

(a) Formation of cholesterol and phospholipids

(b) Formation of lipoproteins

(2) Conversion of carbohydrates and proteins

c. Proteins

(1) Deamination of proteins

(2) Formation of urea

(3) Formation of plasma proteins

- d. Vitamins
    - (1) Liver as reservoir
    - (2) Formation of vitamin A
    - (3) Supply of vitamins D and B_{12}
- e. Storage of iron

3. Excretory
    - a. Bile
        - (1) Secreted from all hepatic cells
        - (2) Secreted into duodenum or stored in gall bladder
        - (3) Bile salts emulsify fat globules
        - (4) Soluble end products of fat digestion
    - b. Bilirubin

B. Definition of Bilirubin
- 1. End product of hemoglobin metabolism
- 2. Chief bile pigment

C. Formation and Excretion of Bilirubin
- 1. Reticuloendothelial system (R.E.S.)

QUESTION: What is the reticuloendothelial system?

ANSWER: The reticuloendothelial system is that part of the body which controls the production and destruction of blood cells and tissue.

- a. Control of production and destruction of cells
- b. Composed of liver, spleen, and lymph nodes

NOTE: Show T 08-280-260-1 and -2.

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c. Lysis of erythrocytes

d. Release of hemoglobin

(1) Heme

(a) Iron

(b) Bilirubin

(2) Globin

2. Bloodstream

NOTE: Show T 08-280-260-3.

a. Protein-bound bilirubin (albumin-bound)

(1) Indirect

(2) Unconjugated

(3) Free

(4) Alcohol soluble

b. Transportation of protein-bound bilirubin to liver

3. Liver

NOTE: Show T 08-280-260-4.

a. Bilirubin diglucuronide

(1) Direct

(2) Conjugated

(3) Water soluble

b. Secretion of bilirubin diglucuronide into the bile

4. Absorption and excretion routes of direct bilirubin

NOTE: Show T 08-280-260-5.

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- a. Bile ducts and gall bladder
- b. Intestines
    - (1) Conversion of direct bilirubin to urobilinogen
    - (2) Reabsorption of some urobilinogen
- c. Kidneys
    - (1) Filter urobilinogen from the blood
    - (2) Excrete urobilinogen as urobilin

D. Occurence of Jaundice

QUESTION: Define jaundice.

ANSWER: Jaundice is the yellow pigmentation noted in the skin and serum of a patient resulting from excessive concentrations of bilirubin in the body.

1. Physiological
    - a. Immature liver
    - b. Increased indirect bilirubin
    - c. UV light or exchange transfusion
2. Hemolytic
    - a. Increased erythrocyte lysis
    - b. Increased indirect bilirubin
3. Hepatocellular
    - a. Liver damage
    - b. Increased indirect bilirubin
4. Obstructive
    - a. Blockage of bile ducts
    - b. Increased direct bilirubin

E. Evelyn-Malloy Serum Bilirubin Determination

1. Principles
    a. Reaction of bilirubin with diazo reagent
    b. Formation of azorubin
    c. Direct reaction - water soluble
    d. Indirect reaction - alcohol soluble
2. Notes and precautions
    a. Timing
    b. Diazo reagent
    c. Light-sensitivity of bilirubin
3. Reagents
    a. Working standard solution
    b. Hydrochloric acid solution
    c. Diazo reagent
        (1) Sodium nitrite solution
        (2) Sulfanilic acid solution
    d. Methanol
4. Specimen of choice
5. Procedural steps
    a. Sample dilution
    b. Serum blank
    c. Direct reading bilirubin
        (1) Sample dilution
        (2) Diazo reagent

(3) One minute reading

d. Total reading bilirubin

(1) Direct mixture

(2) Methanol

(3) Thirty minute reading

6. Calculations

a. Direct

b. Total

c. Indirect

7. Normal serum values

a. Direct - up to 0.3 mg/dl

b. Total - up to 1.5 mg/dl

c. Indirect - up to 1.2 mg/dl

NOTE: Issue M 08-280-260-1, one per student.

F. Questions from Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Functions of the liver

2. Definition of bilirubin

3. Formation and excretion of bilirubin

4. Occurrence of jaundice

5. Evelyn-Malloy serum bilirubin determination

B. Closing Statement: We have briefly discussed the importance of the liver in the body. Care must be taken in performing liver function tests as accurately as possible. As a medical laboratory specialist, you will not diagnose or treat a liver malfunction, but the results of your laboratory tests could mean life or death to a patient. The physician depends on your laboratory results to determine the treatment that he administers.

SECOND THROUGH EIGHTH HOURS

IV. APPLICATION (305 min)

A. Instructors' Activities

1. Step by step, orient the students to the Evelyn-Malloy serum bilirubin determination, to include reagent preparation, procedural precautions, procedural steps, calculations, and sources of test errors.

2. Issue reagents and specimens

3. Supervise student activities

4. Make on-the-spot corrections of students' errors

5. Collect and grade student report sheets

B. Students' Activities

1. Oriented to the Evelyn-Malloy serum bilirubin determination

2. Perform the Evelyn-Malloy serum bilirubin determination as outlined in M 08-280-260-1

3. Report test results to the instructors

4. Record all test data in laboratory notebook

5. Clean up area and equipment as necessary

V. REVIEW (10 min)

A. Questions from Students

B. Review of Noted Errors

1. Student technique

2. Calculations

3. Safety

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C. Closing Statement: All laboratory determinations must be performed with care and accuracy. The serum bilirubin determination is one of the more exacting procedures performed in the clinical chemistry laboratory because it requires a great deal of precise technique.

NINTH HOUR

VI. EXAMINATION (45 min)

A. Directions to Students

1. Put away all lecture notes and texts
2. M 08-280-205-1 and -3 may be used

B. Instructor's Activities

1. Issue quiz sheet, one per student
2. Supervise student activities
3. Clarify any vague points
4. Collect and grade completed quiz sheets

C. Students' Activities

1. Place identification on quiz sheet
2. Answer all questions as appropriate
3. Turn in completed quiz sheet to the instructor

D. Critique

1. Questions from students
2. Reteach instructional material as necessary

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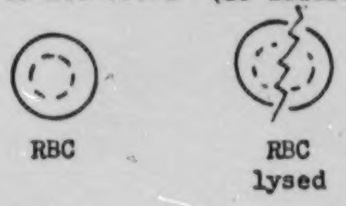
ANNEX A
INSTRUCTIONAL AIDS

Transparencies

T 08-280-260-1 (as illustrated below)



$\longrightarrow$ Hemoglobin released

NOTE: Used in part IIC1 during the first hour.

T 08-280-260-2 (as illustrated below)

$$\begin{tikzcd}
\text{Hemoglobin} \arrow[r] & \text{Heme} + \underbrace{\text{Globin}} \arrow[d] \\
 & \text{(reused)} \\
\text{Heme} \arrow[r] & \text{Bilirubin} + \overbrace{\text{Iron}} \arrow[u]
\end{tikzcd}$$

NOTE: Used in part IIC1 during the first hour.

T 08-280-260-3 (as illustrated below)

$$\text{Bilirubin} \xrightarrow[\text{albumin}]{\text{bound with}} \left[\begin{array}{l}\text{Albumin-bound}\\ \text{Indirect}\\ \text{Unconjugated}\\ \text{Alcohol soluble}\end{array}\right.$$

NOTE: Used in part IIC2 during the first hour.

T 08-280-260-4 (as illustrated below)

INDIRECT BILIRUBIN TRANSPORTED TO THE LIVER

$$\text{Indirect bilirubin} + \text{Glucuronic acid} \longrightarrow \left[\begin{array}{l}\text{Direct}\\ \text{Conjugated}\\ \text{Water soluble}\\ \text{Diglucuronide}\end{array}\right.$$

NOTE: Used in part IIC3 during the first hour.

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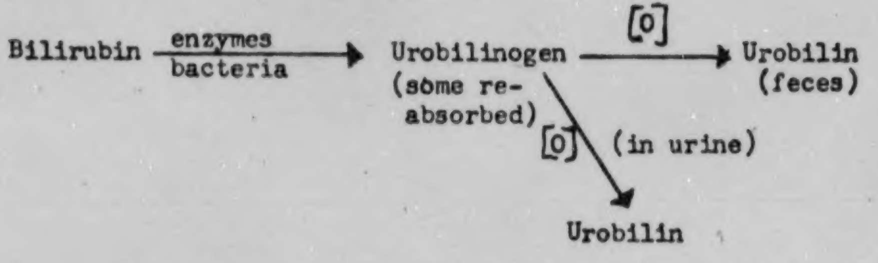
LP 08-280-260
084

T 08-280-260-5 (as illustrated below)

IN THE SMALL INTESTINE


Bilirubin
enzymes
bacteria
Urobilinogen
(some re-
absorbed)
[0]
Urobilin
(feces)
[0]
(in urine)
Urobilin

NOTE: Used in part IIC4 during the first hour.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *IM 08-280-260
LABORATORY SCIENCES DIVISION 084
CHEMISTRY BRANCH

Bilirubin

One of the most important organs of the body is the liver which plays a role in a vast number of the body's processes. Therefore, it is important that some means of measuring the efficiency of liver functions be available. One test for determining some of the liver's functions is based on quantitating a bile pigment called bilirubin.

The functions of the liver can be divided into three basic areas: vascular, metabolic, and excretory. The vascular function involves filtration and detoxification of the blood as it passes through the liver. Approximately 1400 ml of blood per minute passes through the liver. Of this total, 1000 ml enters the portal vein through the sinusoids and the additional 400 ml flow into the sinusoids from the hepatic artery. The process of filtration in the liver takes place in the Kupffer cell filtration system. Kupffer cells cover the sinusoids of the liver and protrude into the flow of blood. These specialized cells phagocytize as much as ninety-nine (99) percent of the bacteria in the portal venous blood that enters completely through the liver sinusoids. This portal venous blood contains bacteria since it drains from the intestines.

Detoxification is the other major vascular function of the liver. The liver is capable of taking many toxic substances and converting them to relatively non-toxic substances. A prime example of a normal toxic waste product is ammonia, which is converted to urea with the addition of carbon dioxide. Alcohol and drugs such as heroin and morphine, which do not naturally occur in the body, are other examples. When ethanol enters the body, the enzyme alcohol dehydrogenase (ADH) in the liver begins converting it to acetaldehyde, an intermediate product. Further breakdown occurs when another enzyme, aldehyde oxidase, reacts with the acetaldehyde to form acetic acid, which is non-toxic in small amounts. When heroin is introduced into the bloodstream, it is transported to the liver and is changed to morphine. Morphine is then combined with glucuronic acid to form a non-toxic compound, morphine glucuronide. A note of caution should be made concerning these drugs and alcohol. The detoxification processes are very slow and limited by the number of non-toxic substances that can be formed.

The metabolic functions of the liver are too varied to detail completely at this time. However, we will discuss the most important areas. Perhaps the most important metabolic function is carbohydrate metabolism, which is

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*This instructor's manuscript supersedes IM 08-280-295, 081.

mainly concerned with the maintenance of the normal blood glucose level (70 to 110 mg/dl). There are three main ways the glucose level is maintained by the liver: the storage of glycogen; the conversion of galactose and fructose to glucose; and gluconeogenesis (the formation of glucose from metabolic substances other than carbohydrates; i.e., amino acids and fatty acids).

Lipid metabolism takes place in almost every cell of the body; however, it is more rapid in the liver than elsewhere. Some authors state that fifty (50) percent or more of the body's various fat interconversions occur in the liver. These interconversions include the formation of large quantities of cholesterol and phospholipids (up to eighty percent of this cholesterol is used to form bile salts and the remainder is transported with the phospholipids in the lipoproteins), the formation of lipoproteins which are absorbed by any cell of the body to help form cell membranes and intracellular structures, and the conversion of large quantities of carbohydrates and proteins to fats.

Protein metabolism is too important to omit because of its close relationship to other metabolic functions of the liver. Protein metabolism involves the deamination of amino acids before they can be used for energy or converted into carbohydrates or fats, the formation of urea, and the formation of plasma proteins.

Vitamins are stored in the liver, which is an excellent reservoir. Vitamin A is formed in larger quantity than any other vitamin found in the liver but normally a large supply of vitamins D and B_{12} are also present. The liver also stores a large portion of the iron in the body and plays a significant role in the coagulation of blood because fibrinogen, prothrombin, and other coagulation factors are found in the liver.

The last major function to be discussed is excretion. There are two main substances excreted from the body by the liver: bile and bilirubin. Bile is a liquid secreted in small quantities from all hepatic cells. Bile, which is either secreted directly into the duodenum or stored in the gall bladder for later use, contains no digestive enzymes but it is important to digestion in that it contains bile salts which help emulsify fat globules and also help render end products of fat digestion soluble so that they can be absorbed from the small intestine.

Bilirubin is the end product of hemoglobin metabolism. It is also the chief bile pigment imparting the bile its yellowish color. The body system that controls the production and destruction of blood cells and tissues is the reticuloendothelial system (R.E.S.). The R.E.S. includes such organs as the liver and spleen and the lymph nodes. When erythrocytes are about 120 days old in the circulating blood, they are lysed by the R.E.S. and hemoglobin is released. This hemoglobin is then broken down to heme and globin and the heme is further broken down to bilirubin and iron. The body reabsorbs the iron and globin for re-use and the bilirubin is left as a waste product.

In the bloodstream, the bilirubin is bound to an albumin form protein bound (albumin bound) bilirubin. Four common synonyms for albumin-bound bilirubin are indirect, unconjugated, free, and alcohol soluble. The indirect bilirubin is then transported via the bloodstream to the liver. After the indirect bilirubin enters the liver, it is changed by glucuronic acid to form bilirubin diglururonide. Direct, conjugated and water soluble are the common sysnonyms for the bilirubin-diglucuronide. This direct bilirubin then leaves the liver in the bile _____ and bile duct. It enters directly into the small intestine or is stored in the gall bladder.

When it enters the small intestine, the direct bilirubin is acted upon by enzymes and bacteria to form an intermediate substance called urobilingen (sterobilinogen). Most urobilinogen converted to form urobilin (sterobilin). Urobilin is the substance which causes the feces to be yellow-brown in color. Some of the urobilinogen is absorbed from the small intestine and is carried by the bloodstream to the kidney where it is oxidized to urobilin in the urine.

When something disrupts this process, a condition known as jaundice develops. Jaundice is the yellow pigmentation seen in the skin and blood serum as a result of the excessive buildup of bilirubin in the body. Jaundice is also easily detected by the yellowish color of the whites of the eyes. It sometimes noted that jaundice is not a cause but the result of some condition of the body. Jaundice may be caused by either a disease or physiological blockage or immaturity of the liver. There are four major types of jaundice: physiological, hemolytic, hepatocellular, and obstructive.

Physiological jaundice is found in newborn infants and is due to an immature liver which is usually self-correcting as the infant matures. Indirect bilirubin has increased in this type of jaun dice where the immature liver cannot conjugate to direct bilirubin as quickly as it builds up in the blood. In such cases of physiological jaundice, one of two treatments is usually administered. Laboratory test results usually help the physician decide whether the infant is to be treated with ultraviolet light or with an exchange transfusion. With UV light treatment, the infaat is continuously exposed to fluorescent light source for six ____. The indirect bilirubin is photodecomposed to products that are effective without conjugation, in the bile and urine. This type of treatment is not widely accepted in the United States because of disagreements among authors about the long-term effects of exposure to UV light and whether or not the decomposition products are toxic. In very severe cases of physiological jaundice, an exchange transfusion is utilized. This procedure involves

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the removal of blood from the jaundiced infant while replacing its blood with fresh donor blood.

Hemolytic jaundice is caused by excessive lysis of erythrocytes. Excessive lysis may result from a transfusion reaction or hemolytic disease of the newborn. As is the case with physiological jaundice, indirect bilirubin increases. The increase is due to the inability of the liver to convert such an excessive amount of indirect bilirubin to direct bilirubin.

Hepatocellular jaundice results from damage to the liver. Damage may occur in diseases such as cirrhosis, severe acute hepatitis, or other hepatic diseases. Indirect bilirubin is increased since the damaged liver cells cannot convert it to direct bilirubin.

Obstructive jaundice is caused by an obstruction to the bile ducts. There is an increase of direct bilirubin because of the obstruction (the bilirubin has already been conjugated in the liver).

In the Evelyn-Malloy serum bilirubin determination, bilirubin reacts with diazo reagent to form red-violet azobilirubin (azorubin). The direct bilirubin is water soluble and reacts with the diazo reagent in an aqueous solution. Indirect bilirubin is almost insoluble in water and methanol must be added to the test mixture to release the indirect bilirubin for the reaction. In the test, timing is important. The direct reading must be taken at exactly one minute to eliminate possible error from small amounts of the indirect bilirubin reacting. The diazo reagent is very unstable and the sodium nitrite must be added to the sulfanilic acid just before use. Bilirubin is light-sensitive; the specimen must be protected from strong light sources and stored in the dark if the test is not performed shortly after the specimen is collected.

Reagents used for the Evelyn-Malloy test include a 0.4 mg/dl (w/v) working standard solution, a 1.5 mg/dl (w/v) hydrochloric acid solution (diazo blank), diazo reagent made of sodium nitrite and sulfanilic acid, and absolute methanol. Serum is the specimen of choice. To set up the test, a 1:10 dilution of the serum is made with water and the dilution is halved for use as the "test" and the "serum blank." The hydrochloric acid solution is added to the serum blank cuvet and the diazo reagent is added to the test cuvet. The direct reading is taken at exactly one minute after the addition of the diazo reagent. Methanol is added to both cuvets and after thirty minutes, the total reading is taken. Concentrations for the direct and total bilirubin values are obtained from a calibration curve or by calculation with a standard subjected to the test procedure. Indirect bilirubin values are algebraically derived from the total and direct bilirubin values (total minus direct equals indirect). Normal serum ranges of bilirubin concentrations are up to 0.3 mg/dl direct bilirubin,

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up to 1.5 mg/dl total bilirubin and up to 1.2 mg/dl indirect bilirubin.

During this period of instruction, we have discussed three basic functions of the liver, the definition of bilirubin, the formation and excretion of bilirubin, the types of jaundice, and the Evelyn-Malloy serum bilirubin method.

We have briefly discussed the importance of the liver in the body. Care must be taken in performing liver function tests as accurately as possible. As a medical laboratory specialist, you will not diagnose or treat a liver malfunction but the results of your laboratory tests could mean life or death to a patient. The physician depends on your laboratory results to determine the treatment that he administers.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
CHEMISTRY BRANCH

*M 08-280-260-1
113

Serum Bilirubin Determination (Evelyn-Malloy)

A. PRINCIPLE

Sodium nitrite is added to sulfanilic acid to form diazo reagent. The diazo reagent couples with serum bilirubin to form red-violet colored azobilirubin which is quantitated colorimetrically.

B. REAGENTS:

1. Bilirubin working standard (0.4 mg/dl).

2. 1.5 ml/dl HCl.

3. Sodium nitrite (0.5 g/dl).

4. p-Sulfanilic acid (0.1 g/dl) -- 1 gram of p-sulfanilic acid. Q.s. to 1 liter with 1.5 ml/dl HCl.

5. Diazo reagent.

    a. Into a 50 ml Erlenmeyer flask add the following reagents in order:

    b. 10 ml of 0.1 g/dl sulfanilic acid (use 10 ml serological pipet)

    c. 0.3 ml of 0.5 g/dl sodium nitrite solution. (use 1 ml serological pipet)

    d. Mix by swirling gently.

    NOTE: Prepare diazo reagent just before use. The reagent is relatively unstable at room temperature. If the diazo turns yellow it must be discarded and prepared again.

6. Methanol (absolute) -- analytical grade.

    NOTE: As a substitute for absolute methanol, prepare 40 ml/dl isopropyl alcohol. Do not interchange methanol and isopropyl alcohol in the procedure. Use either methanol or isopropyl alcohol but not BOTH in the same set of determinations.

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*This mimeo supersedes M 08-280-260-1, 063.

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M 08-280-260-1
113

C. NOTES

1. Diazo reagent must be used within 10 minutes after preparation, since it is unstable over longer periods of time.

2. This procedure measures only TOTAL bilirubin. To measure direct bilirubin, using the same blanking procedure, read the "T" cuvets at EXACTLY one minute after adding the diazo reagent and before adding the methanol.

3. Indirect bilirubin is determined by substracting the direct bilirubin from the total bilirubin concentrations. Therefore, total bilirubin minus direct bilirubin is equal to the indirect bilirubin.

4. Serum bilirubin is not stable and deteriorates rapidly in the presence of certain wavelengths of light. If storage is necessary, be sure to refrigerate the specimens and protect from light.

5. A serum blank must be prepared with each unknown to compensate for pigments present in the serum.

6. HCl is added to the blank to prevent turbidity.

7. After adding methanol, if the solution shows signs of turbidity, place the test tube or cuvet in a 37° C. water bath; this will clear the solution, unless the specimen is lipemic (fat-containing).

8. Serum specimens must be clear and free of hemolysis. Because of the presence of lipids, specimens taken soon after a meal may be unsuitable.

9. If kept in total darkness, serum bilirubin is stable at room temperature for approximately 48 hours; at 4° C for 4 to 7 days; and for approximately 3 months at minus 20° C.

D. NORMAL VALUES

1. Direct biliruin -- up to 0.3 mg/dl

2. Indirect bilirubin -- up to 1.2 mg/dl

3. Total bilirubin -- up to 1.5 mg/dl

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E. CLINICAL SIGNIFICANCE

1. Physiological jaundice
   - a. Indirect bilirubin increased
   - b. Due to immature liver
2. Hepatocellular jaundice
   - a. Indirect bilirubin increased
   - b. Due to liver cell damage
3. Obstructive jaundice
   - a. Direct bilirubin increased
   - b. Due to physical blockage of bile ducts
4. Hemolytic jaundice
   - a. Indirect bilirubin increased
   - b. Due to excessive RBC breakdown

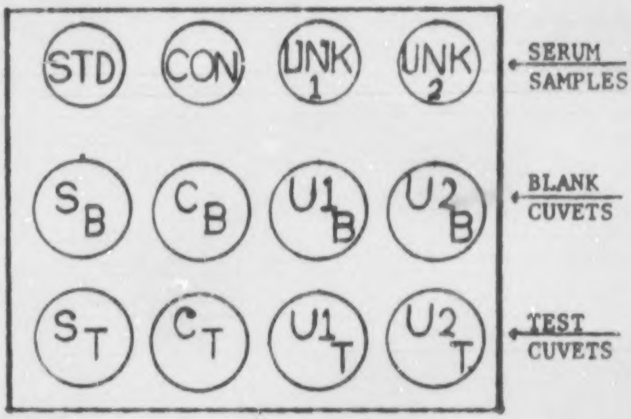
B. PROCEDURE

1. Each sample has its own blank cuvet, therefore, label TWO cuvets for each unknown, standard and control. One set is labeled "B" for the blanks, while the other set of cuvets is labeled "T" for the test cuvets.

   EXAMPLE: Your rack should be set up in accordance with the diagram below.

LABEL LEGEND

S_B = Standard blank
C_B = Control blank
$Number_B$ = Unknown blank
S_T = Standard test
C_T = Control test
$Number_T$ = Unknown test
STD = Standard
CON = Control
UNK = Unknown



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2. Pipet 1 ml of each standard, control and unknown into its "B" cuvet. (use a 1 ml Ostwald-Folin pipet)

3. Add 9 ml deionized water to each "B" cuvet and mix well. (use a 10 ml serological pipet)

4. Transfer 5 ml of the diluted sample from the "B" cuvet to the corresponding "T" cuvet. (use a 5 ml transfer pipet)

5. Pipet 1 ml of 1.5 ml/dl HCl to ALL "B" cuvets and mix well. (use a 5 ml serological pipet)

6. Prepare fresh diazo reagent. (see note 5 in section B of the procedure)

7. Pipet 1 ml of fresh diazo reagent into each "T" cuvet and mix well. (use a 5 ml serological pipet)

8. Add 5 ml methanol to ALL "B" and "T" cuvets and mix well using a Parafilm cover. Invert at least 3 times to insure uniform mixing. (use a 10 ml serological pipet)

9. Incubate samples at room temperature for 30 minutes.

SUMMARY OF STEPS 1 through 9

	"B" Cuvets	"T" Cuvets
Diluted sample	5 ml	5 ml
1.5 percent HCl	1 ml	-
Diazo reagent	-	1 ml
Methanol (or isopropyl alcohol)	5 ml	5 ml

10. Set spectrophotometer at 540 nm after allowing an adequate warm-up time for the instrument.

11. After the 30 minute incubation, zero the spectrophotometer. Adjust 100 percent transmittance (100% T) using the "B" cuvets for each individual test. Read the corresponding "T" cuvet and record the absorbancy.

12. Calculate control and unknown concentrations using the formula in part "G" of this determination.

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G. CALCULATIONS

$$\frac{A_S}{C_S} = \frac{A_U}{C_U}$$

C_S = 0.4 mg/dl bilirubin
A_S = absorbancy of bilirubin standard
A_U = absorbancy of unknown
C_U = concentration of unknown (mg/dl)

H. REFERENCES:

1. TM 8-227-6, pp 9-7 to 9-8.

2. Tietz, Norbert W. Fundamentals of Clinical Chemistry. 1st ed. Philadelphia: W. B. Saunders Company, 1970, pp743-756.

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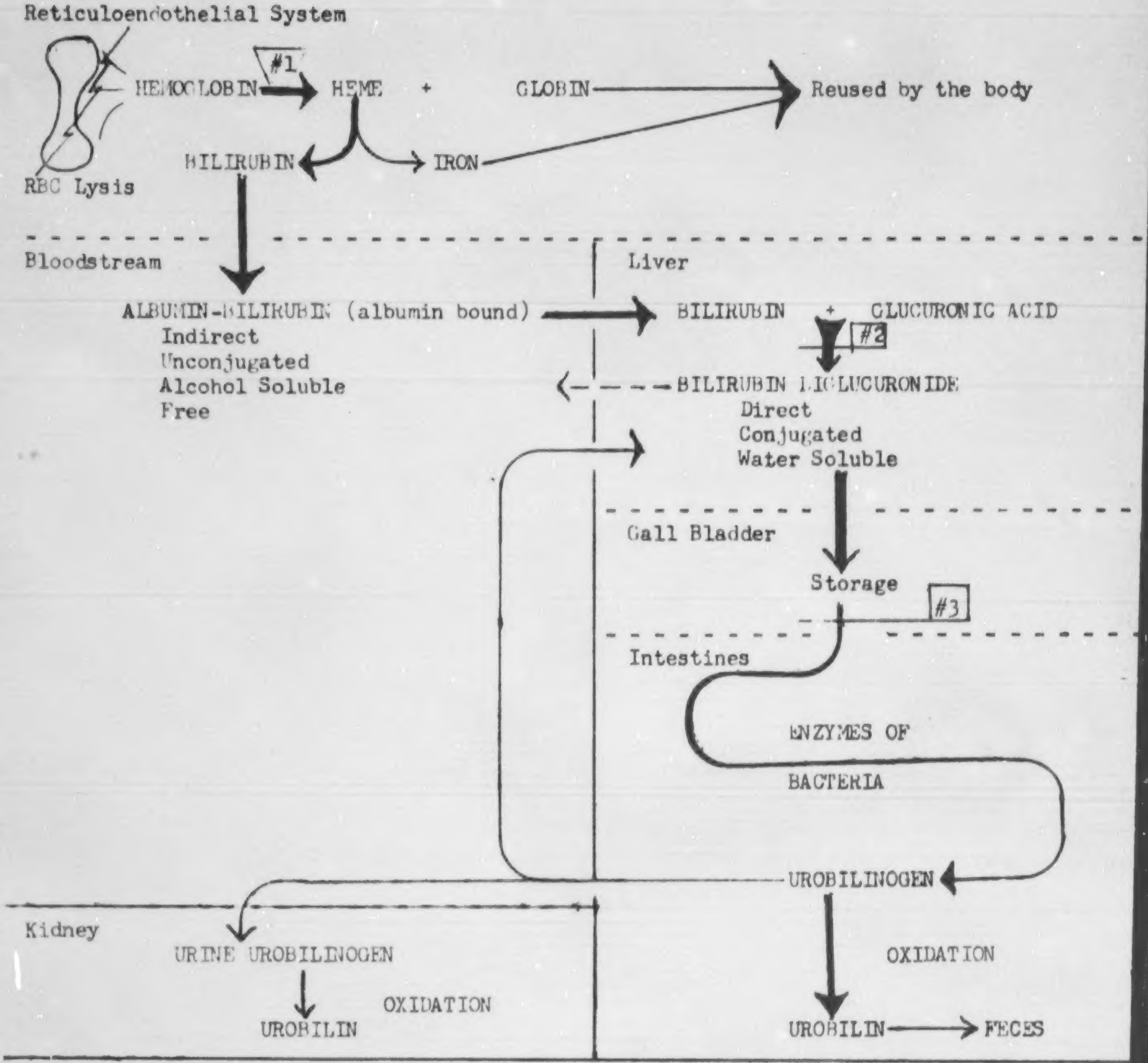
ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
CHEMISTRY BRANCH

M 08-280-260-3
095

FORMATION AND EXCRETION OF BILIRUBIN


Reticuloendothelial System
#1
HEMOGLOBIN
HEME
+
GLOBIN
Reused by the body
BILIRUBIN
IRON
RBC Lysis
Bloodstream
Liver
ALBUMIN-BILIRUBIN (albumin bound)
Indirect
Unconjugated
Alcohol Soluble
Free
BILIRUBIN
+
GLUCURONIC ACID
#2
BILIRUBIN DIGLUCURONIDE
Direct
Conjugated
Water Soluble
Gall Bladder
Storage
#3
Intestines
ENZYMES OF
BACTERIA
UROBILINOGEN
Kidney
URINE UROBILINOGEN
OXIDATION
UROBILIN
OXIDATION
UROBILIN
FECES

	PROBLEM		PRIMARY TYPE OF BILIRUBIN INCREASED
#1	Increased RBC lysis	- - - - - - -	Unconjugated
#2	Conjugation failure	- - - - - - -	Unconjugated
#3	Obstruction	- - - - - - -	Conjugated

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-265
LABORATORY SCIENCES DIVISION 084
CHEMISTRY BRANCH

Enzymes (7 hours)

COURSES PRESENTED TO: 311-92B20, Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES:

TM 8-227-6, chap 12
Bergmeyer, H.U. Methods of Enzymatic Analysis. New York: Academic Press, 1963, p 853.
Davidsohn, I., and Henry, J.B., ed. Todd-Sanford Clinical Diagnosis by Laboratory Methods. 14th ed. Philadelphia: W.B. Saunders Company, 1969, p 728.
Karlson, P. Introduction to Clinical Biochemistry. 3rd ed. New York: Academic Press, 1969, pp 321-322.
Karlson, P. Introduction to Modern Biochemistry. New York: Academic Press, 1969, pp 71-95.
Oser, B.L., ed. Hawk's Physiological Chemistry. New York: McGraw-Hill, 1965, pp 461-463.
Schmidt, E. Fundamentals of Diagnostic Enzymology. San Francisco: Boehringer Mannheim Corporation, 1968, p 29.
Tietz, N.W., ed. Fundamentals of Clinical Chemistry. Philadelphia: W. B. Saunders Company, 1970, pp 409-415.

STUDY ASSIGNMENT: After class, review M 08-280-265-1 and -2.

STUDENT UNIFORM AND EQUIPMENT: Slide rule

TOOLS, EQUIPMENT, AND MATERIALS: M 08-280-265-1 issued one per student at the beginning of the fourth hour. M 08-280-265-2 issued one per student at the beginning of the first hour.

PERSONNEL: 1st thru 3rd and 7th hours, one instructor (MOS 92B4H); 4th thru 6th hours, eight instructors (MOS 92B4H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, eraser, overhead projector, T 08-280-265-1 (see Annex A)

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture (1st thru 3rd hours), PE (4th thru 6th hours), EX (7th hour)

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*This lesson plan supersedes LP 08-280-250, 081.

FIRST THROUGH THIRD HOURS

NOTE: Issue M 08-280-265-2, one per student. Have students take lecture notes on this mimeo.

I. INTRODUCTION (10 min)

A. Opening Statement: Catalysts in the body are identified as enzymes which are responsible for bringing about almost every chemical reaction involved in the process of life. Consequently, because of their importance to body function, measurements of enzymes serve as important, diagnostic tools in the identification of various diseases.

B. Objectives

1. Define enzyme, substrate, and product.
2. List the factors which influence enzyme activity.
3. Differentiate between two principles of measuring enzyme activity.
4. Explain the use of enzymes as a diagnostic tool.
5. Define an international unit of enzyme activity.
6. Describe some of the factors influencing the action of amylase.
7. With practice, correctly perform an amylase determination within acceptable accuracy limits.

C. Class Procedure and Lesson Tie-in

1. First three hours are lecture.
2. Second three hours consist of a practical exercise on the measurement of the enzyme amylase by the iodometric method.

II. EXPLANATION (120 min)

A. Chemical Nature

1. Organic catalyst

a. Active in small amounts

b. Influences rate of reaction

c. Recovered after the reaction

2. Protein--usually conjugated

QUESTION: What is conjugated protein?

ANSWER: A chain of amino acids and a prosthetic group.

a. Native state--functional

b. Denatured state--non-functional

3. Specific nature

a. Use a specific substrate

NOTE: Define substrate as the substance or substances upon which an enzyme acts.

b. Produce specific product(s)

B. Cofactors

1. Combine with enzyme

2. Make enzyme functional

3. Examples

a. Coenzymes--vitamins

b. Metal ions

C. Function

1. Active sites

a. Sites of chemical reaction

b. Provide specificity

2. Enzyme-substrate complex

3. General equation ($E + S \rightleftharpoons ES \rightarrow E + P$)

NOTE: Show T 08-280-265-1. See Annex A.

D. Nomenclature

1. Common names
    a. Oldest method
    b. Examples
        (1) Pepsin
        (2) Trypsin
        (3) Chymotrypsin
2. Substrate based names
    a. Substrate plus ending "-ase"
    b. Examples
        (1) Urease (from urea)

QUESTION: What is the name of the enzyme that uses maltose (malt sugar) as its substrate?

ANSWER: Maltase.

(2) Maltase (from maltose)

(3) Amylase (from starch--amylum in Latin)

3. Reaction based names
    a. Reaction plus ending "-ase"
    b. Examples
        (1) Lactic acid dehydrogenase (removes H+)
        (2) Carbonic anhydrase (removes H_2O)
        (3) Glutamic pyruvate transaminase (transfers an amine group)

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4. IUB system (International Union of Biochemists)
    - a. Numerical system
    - b. Reaction based
    - c. Examples
        - (1) 1.1.3.4 - (Glucose oxidase) D-glucose: O_2 oxidoreductase
        - (2) 2.6.1.1 - (Glutamic oxaloacetic transaminase) aspartate: 2 - oxoglutarate aminotransferase
        - (3) 3.2.1.1 - (Amylase) α-1, 4-glucan 4-glucanohydrolase

E. Factors Influencing Enzyme Activity

NOTE: See Annex B for graphic illustrations of these effects.

1. Temperature
    - a. Increased temperature - increased rate
    - b. Denaturation temperature - about 60° C

QUESTION: What happens to egg white when fried?

ANSWER: It coagulates and turns white.

- (1) Coagulation
- (2) Precipitation
- (3) Irreversible

- c. Inactivation temperature
    - (1) Freezing - minus 20° C or less
    - (2) Reversible

2. pH
    - a. Optimum pH - 5 to 7

NOTE: Each enzyme has its own optimum pH.

- b. Maintenance by buffer systems
- c. Enzyme inactivation

        (1) Changed active site
        (2) Denaturation

3. Enzyme concentration

   Rate proportional to concentration

4. Substrate concentration
   a. Rate proportional to concentration up to V_{max}
   b. Concentration greater than V_{max}
      (1) Rate decrease - crowding effect
      (2) Available enzyme converted to ES complex
5. Time
   a. Substrate decreased inversely per unit time
   b. Products increased proportionally per unit time
6. Activators
   a. Required by many enzymes
   b. Make enzyme functional
   c. Examples
      (1) Zn^{++} for carbonic anhydrase
      (2) Ca^{++} for blood clotting enzymes
      (3) Mg^{++} for glycolytic enzymes
7. Inhibitors
   a. Destruction of enzyme activity
      (1) Change or block active sites
      (2) Destroy protein structure
   b. Examples

(1) Heavy metals (lead, mercury, copper)

(2) Organic solvents (alcohol, acetone)

(3) Detergents

(4) Anticoagulants

QUESTION: What property of anticoagulants, such as EDTA and sodium citrate, makes them enzyme inhibitors?

ANSWER: They bind calcium ions (the activator of blood clotting enzymes).

F. Measuring Enzyme Activity (Reaction Rate)

NOTE: Enzyme activity is simply defined as the amount of substrate converted or the amount of products produced per unit time.

1. Approaches
    a. Measure remaining substrate
    b. Measure products formed
    c. Measure hydrogen acceptors (NAD) formed or converted
2. Activity units
    a. Advantages
        (1) Unnecessary to isolate individual enzymes
            (a) Difficult to isolate - similar to other enzymes
            (b) Found in very small amounts
        (2) Measures only native or active enzymes
        (3) Measures turn-over rate - enzyme reversibility
    b. Units of report
        (1) International Unit (IU)
            (a) Definition - That amount of enzyme activity which converts one micromole of substrate or produces one micromole of product per minute under standard conditions.

NOTE: All of the rate-influencing factors must be carefully controlled and standardized for the units of report to be meaningful.

(b) Usage - ImU/ml of serum

NOTE: Unnecessary for students to memorize the following units.

(2) Somogyi units

(3) Karmen units

(4) King-Armstrong units

G. Enzymes as a Diagnostic Tool

1. Tissue specificity of enzymes

2. Release of enzymes by damaged tissue

a. Characteristic of the damaged tissue

b. Elevation of serum enzyme levels

c. Elevation proportional to extent of tissue damage

3. Examples

NOTE: Unnecessary for students to memorize these examples.

a. Amylase

(1) Tissue sources

(a) Pancreas

(b) Salivary glands

(2) Serum levels elevated

(a) Pancreatitis

(b) Mumps

b. Glutamic oxaloacetic transaminase (GOT)

(1) Tissue sources

(a) Heart

(b) Liver

(2) Serum levels elevated
    (a) Heart attacks
    (b) Liver disease

c. Creatine phosphokinase (CPK)
    (1) Tissue sources
        (a) Heart
        (b) Muscles
    (2) Serum levels elevated
        (a) Heart attack
        (b) Muscular dystrophy

d. Glutamic pyruvate transaminase (GPT)
    (1) Tissue source - liver
    (2) Serum levels elevated - liver diseases

H. Amylase
    1. General characteristics
        a. Hydrolytic reaction
        b. Substrate
            (1) Starch
            (2) Glycogen
        c. Products
            (1) Dextrins (short-chained polysaccharides)
            (2) Maltose
    2. Sources

QUESTION: Where in the body is amylase produced?

ANSWER: In the pancreas and the salivary glands.

- a. Salivary glands
- b. Pancreas

3. Activity factors
    - a. Temperature - 40 to 50° C optimum
    - b. pH - 6.9 to 7.2 optimum
    - c. Activators
        - (1) Chloride ions
        - (2) Calcium ions
    - d. Inhibitors
        - (1) Acids
        - (2) Anticoagulants
        - (3) Others

QUESTION: What other substances act as inhibitors to amylase?

ANSWER: Organic solvents, detergents, and heavy metals.

- (a) Organic solvents
- (b) Detergents
- (c) Heavy metals

    - e. Time
    - f. Substrate concentration

4. Laboratory determinations
    - a. Approaches

QUESTION: What are two methods of measuring enzyme activity (reaction rate)?

ANSWER: Measure the amount of product formed or the amount of substrate remaining after a unit of time.

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- (1) Measure products formed
- (2) Measure remaining substrate

b. Methods

- (1) Saccharogenic

  Measures sugar formed

- (2) Amyloclastic

  Measures time required to convert a given amount of substrate

- (3) Iodometric

  Measures remaining substrate

I. Amylase Values

1. Normal serum levels
   - a. 0.06 to 0.34 ImU/ml
   - b. 60 to 160 Somogyi units/dl
2. Decreased serum values
   - a. Little clinical significance
   - b. Immature pancreas
3. Increased serum values
   - a. Acute pancreatitis
   - b. Pancreatic duct obstruction
   - c.. Mumps

J. Questions From Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Chemical nature

2. Cofactors
3. Function
4. Nomenclature
5. Factors influencing enzyme activity
6. Measuring enzyme activity
7. Enzymes as a diagnostic tool
8. Amylase
9. Amylase values

B. Closing Statement: Enzyme determinations are requested with increased frequency by the physician. It is essential for you as a laboratory specialist to be familiar with the principles and pitfalls of enzymatic tests so as to be able to perform these tests with accuracy and precision.

FOURTH THROUGH SIXTH HOURS

VI. APPLICATION (135 min)

NOTE: Issue M 08-280-265-1, one per student.

A. Instructors' Activities

1. Issue reagents and specimens
2. Supervise students' activities
3. Make on the spot corrections of students' errors
4. Grade students' results

B. Students' Activities

1. Satisfactorily perform the iodometric amylase determination
2. Report test results to instructors
3. Clean up area and equipment

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VII. CRITIQUE (10 min)

A. Questions From Students

B. Discussion of Errors

1. Technique

2. Calculations

3. Safety

C. Closing Statement: As you have experienced, obtaining accurate and reproducible test results from enzymatic determinations is difficult. As medical laboratory specialists, it will be your responsibility to report the results of enzymatic tests accurately and efficiently to the physician. Therefore, it is necessary to learn proper laboratory technique to accomplish this mission.

SEVENTH HOUR

VIII. EXAMINATION (45 min)

A. Directions To Students

1. Remove all lecture notes and texts

2. M 08-280-205-1 and -3 may be used

3. Place identification on the quiz sheet

B. Instructor's Activities

1. Issue quiz sheet, one per student

2. Supervise students' activities

3. Clarify any vague points

4. Collect and grade completed quiz sheets

C. Students' Activities

1. Place identification on quiz sheet

2. Answer all questions as appropriate

3. Turn completed quiz sheet in to the instructor

D. Critique

1. Questions from students

2. Reteach instructional material as necessary

ANNEX A
INSTRUCTIONAL AIDS

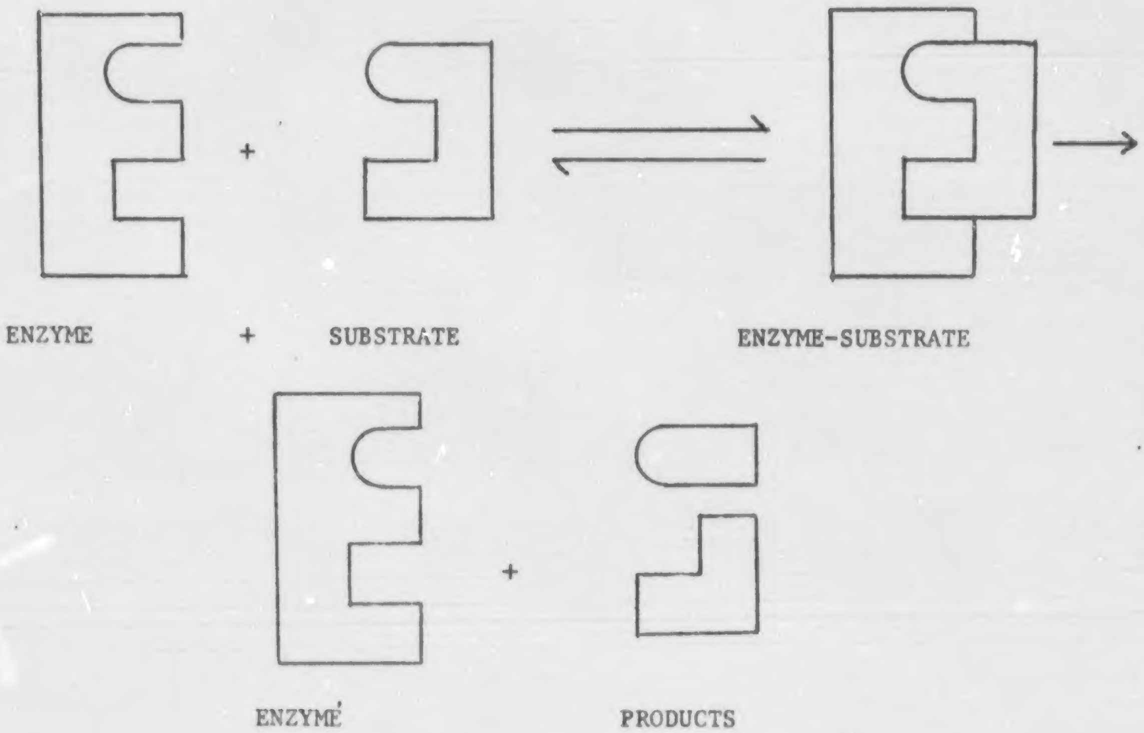
Transparency

T 08-280-265-1 (73-5290) as illustrated below.

1. $E + S \rightleftharpoons ES \longrightarrow E + P$

NOTE:
E = enzyme
S = substrate
P = product

2.


+
ENZYME
+
SUBSTRATE
ENZYME-SUBSTRATE
+
ENZYME
PRODUCTS

NOTE: Used in part IIC during the first hour.

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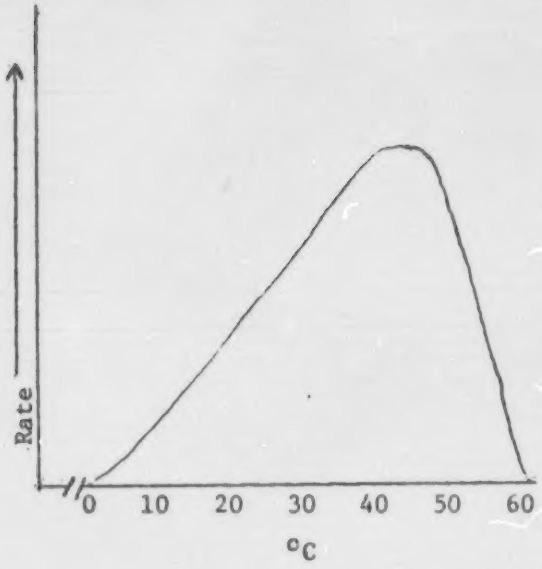
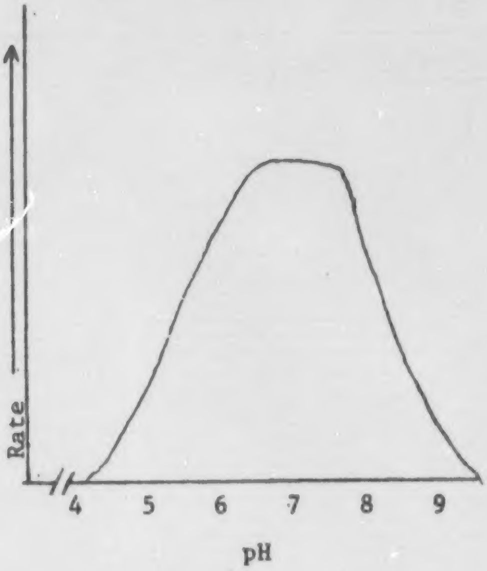
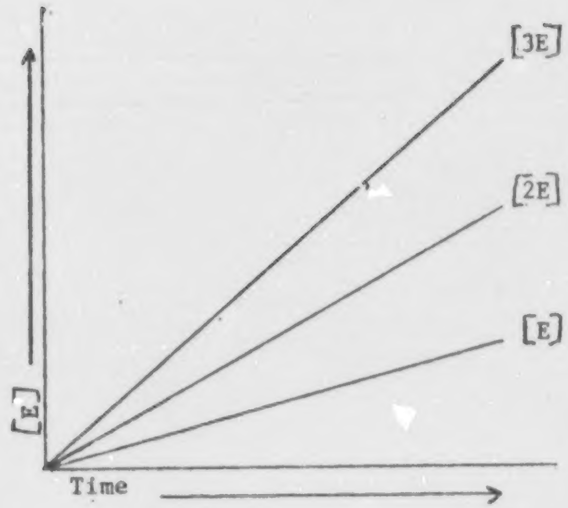
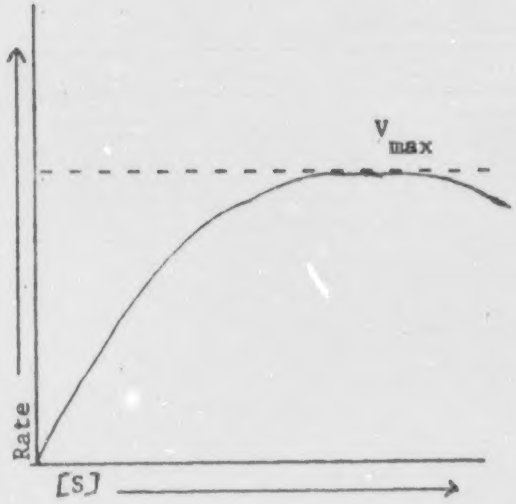
ANNEX B
INSTRUCTIONAL AIDS

Chalkboard Drawing









NOTE: Used in part IIE During the second hour.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *IM 08-280-265
LABORATORY SCIENCES DIVISION 084
CHEMISTRY BRANCH

Enzymes

Catalysts in the body are identified as enzymes which are responsible for bringing about almost every chemical reaction involved in the process of life. Consequently, because of their importance to body function, measurements of enzymes serve as important, diagnostic tools in the identification of various diseases.

Enzymes are organic catalysts. Catalysts function in very small concentrations, usually influencing the rate of a chemical reaction by increasing the reaction rate. They are not consumed by the reaction that they catalyze. Therefore, catalysts can be completely recovered in their original unaltered form after the reaction has taken place and are thus reusable. Enzymes may be composed of simple proteins, these proteins consist of only amino acids. However, they are usually composed of conjugated protein, which is protein containing a prosthetic group in addition to amino acids. Enzymes are very active and functional in their native (natural) state, but many chemical and physical agents can change an enzyme's shape (spatial arrangement), converting it to a nonfunctional, denatured state. Because of an enzyme's complex structure, the reactions it catalyzes are of a very specific nature, i.e., it only reacts with a specific substance, the substrate, to produce a specific product.

Many enzymes are not functional by themselves in a pure form, but need cofactors to aid their activity. These cofactors may be in the form of a firmly bound prosthetic group or in the form of a weakly attached, readily dissociable group. Cofactors may be in the nature of either coenzymes, organic molecules with a vitamin base, or as simple inorganic metal ions. Enzymes are able to function as very specific catalysts because of their complex structure. There is one location on an enzyme that has a specific spatial configuration with specific chemical properties. This site, known as the active site, is the site with which the substrate molecule must come in contact in order for a chemical reaction to take place. Because the active site is so specific in shape and chemical properties, it only catalyzes a specific reaction with a specific substrate to form a specific product. Enzymes function by briefly combining with the substrate molecule at its active site, forming what is called an enzyme-substrate complex. While combined in this enzyme-substrate complex, strains are placed on the bonds that hold the substrate molecule together, causing a reaction to take place. The products are then released from the active site of the enzyme. This reaction can be represented by the equation $E + S \rightleftharpoons ES \rightarrow E + P$. Note by the double arrows that the reaction can go in either direction, depending on the equilibrium that exists, the enzyme being recovered unchanged after the reaction.

A number of methods have been employed for naming enzymes. The oldest

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*This instructor's manuscript supersedes IM 08-280-250, 081.

is the use of a common name, such as pepsin, trypsin, and chymotrypsin. These names usually tell us very little, if anything, about the reaction that the enzyme catalyzes. With the discovery of many additional enzymes the use of common names became very confusing. For this reason, a systematic method of naming enzymes was developed using the name of the substrate as a base for the name and adding the ending "-ase." For example, the enzyme that uses urea as its substrate is named urease. The enzyme that catalyzes a reaction with maltose (malt sugar) is called maltase. Amylase, therefore, is the enzyme that uses starch (amylum is Latin for starch) as its substrate. As knowledge about enzymatic reactions increased it was learned that more than one enzyme could use the same substrate with the production of different products. It now became necessary to include the name of the reaction as well as the name of the substrate when naming enzymes. In this system of nomenclature, it is conventional to add the ending "-ase" to the name of the reaction. For example, the enzyme that removes hydrogen ions from the lactic acid molecule is named lactic acid dehydrogenase. The enzyme that removes a water molecule from carbonic acid is named carbonic anhydrase. The enzyme glutamic pyruvate transaminase, therefore, would be an enzyme that transfers an amine group between glutamic acid and pyruvate. However, with still more enzymes being named and the similarity of some of the reactions catalized on the same substrate, confusion reigned. To further systematize the naming of enzymes, the International Union of Biochemists (IUB) established the IUB system. It is also a reaction based system. However, numbers are used to catalog each type of reaction. The designated number consists of four numbers each separated by a decimal. The first number designates the general type of reaction involved. It can range from 1 to 6, representing oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases, respectively. Each of the three numbers that follow further subdivide each of these categories into more specific reactions in an outline fashion. For example, glucose oxidase is represented by the number 1.1.3.4. The first 1 indicates that it belongs to the group of enzymes called oxidoreductases which catalyze oxidation reduction reactions. The second 1 indicates that it works on a secondary alcohol group. The 3 indicates that the reaction uses O_2 as the hydrogen acceptor. The 4 represents a specific enzyme that catalyzes the reaction. Glutamic oxaloacetic transaminase is cataloged as 2.6.1.1. The 2 indicates that it is a transferase; the 6, the group being transferred is a nitrogen containing group; the first 1, the nitrogen containing group is an amino group; the final 1, a more specific reaction. 3.2.1.1 for amylase represents a hydrolase (3), that splits glycosides (2) by breaking the glycoside bonds (first 1).

There are many factors which influence the rate of an enzymatic reaction. Increasing temperature increases the rate of enzymatic reactions up to an optimum temperature of between 40 and 50° C (a few reach a maximum rate near 60° C). It should be remembered that each enzyme has its own optimum temperature. Generally, temperatures above 60° C serve to denature enzymes by changing the spatial configuration of the protein and hence the enzyme. This denaturation may result in the coagulation (as in the egg white of a fried egg) or precipitation of the enzyme, irreversible reactions. Enzymes may also be inactivated by freezing temperatures, (-20° C or less) since the free movement of molecules throughout a solution is inhibited. This inactivation is generally reversible.

Another important rate-influencing factor is pH. Again, each enzyme has its own optimum pH range. Most enzymes have an optimum pH around neutral or just to the acid side of neutral (pH 5 to 7). In performing enzymatic determinations it is important to maintain a constant pH by using buffer systems, since changes in pH can inactivate enzymes by either causing chemical changes in the active site or by changing the spatial configuration of the protein molecule.

The amount of product formed by an enzymatic reaction in a given amount of time is proportional to the enzyme concentration, $[E]$, provided that all other factors are constant. Due to this fact, we are able to measure enzyme activity.

Substrate concentration also affects enzyme activity. As substrate concentration, $[S]$, is increased, enzyme activity also increases up to a maximum limit often designated as V_{max} (point of maximum reaction velocity). As the $[S]$ is further increased above the concentration needed for V_{max}, the reaction rate is decreased due to the crowding effect of the substrate competing for the active site.

Time acts indirectly upon enzyme activity by changing some of the other rate-influencing factors. As time increases, substrate concentration decreases correspondingly. Another aspect of time is the fact that as time increases, the concentration of products also increases. If these products are either basic or acidic, they may change the pH of the solution over a period of time, thus changing enzyme activity.

Activators are needed by many enzymes to make them functional. When performing an enzyme determination, you must be mindful of the specific activators required by the enzyme you are working with and insure that the activators are present in the proper quantities to insure maximum enzyme activity. Examples of activators include Zn^{++}, needed to activate carbonic anhydrase; Ca^{++} for the blood clotting enzymes; and Mg^{++} for some of the glycolytic enzymes.

There are also substances that act as inhibitors and destroy enzyme activity. Examples of inhibitors include heavy metals, such as lead, mercury, and copper; organic solvents, such as alcohol and acetone; detergents; and anticoagulants of the type that bind activators such as Ca^{++}.

There are several approaches to measuring enzymatic reaction rate. The two most important methods are measuring remaining substrate or measuring increasing product. A third method measures a change in hydrogen acceptors such as NAD and NADP, and can be used in measuring the activity of oxidoreductases that use these molecules as hydrogen acceptors or donors.

Enzyme activity is reported in activity units rather than concentration. This eliminates the need to isolate indivdual enzymes, which is most difficult because of their low concentration and chemical and physical similarity to other enzymes. By using activity units the technician only measures enzymes in their native, active state. He need not concern himself with any denatured or inactive forms that may be present. Most important, he is able to measure the turn-over rate or reusability of an enzyme. Enzymes, being catalysts, are not consumed by the reaction, so the number of reactions that an individual enzyme molecule can catalyze in a given period of time is also important.

There are a number of units of report used to express enzyme activity. The most standardized activity unit is the International Unit (IU) established by the International Union of Biochemists. One IU is defined as the amount of enzyme activity that can convert one micromole of substrate into product per minute (1 µM/min) under standard conditions. Results in the clinical laboratory are usually expressed in International milliUnits per ml of serum (ImU/ml). Previously, many researchers defined their own units of activity due to the lack of a standardized unit. For this reason we quite often see other units of report such as Somogyi, Karmen, Bodansky and King-Armstrong units. Regardless of the units used, the test must be standardized and followed exactly. Without standardization,a comparison of results obtained between different sets of determinations could not be made. Comparisons could only be made between the samples in a single determination, provided that all the tubes were treated exactly the same. If a different time, temperatue, pH, or [S] were used, too many variables would be affected to obtain a meaningful comparison.

Enzymes serve as valuable diagnostic tool owing to the fact that many organs contain enzymes specific to that organ and not found in other body tissues. If a tissue becomes damaged or diseased the fluids, including enzymes, within that tissue will be released. Their increased concentration in the blood then serves as an index of the degree of tissue damage, since their release usually will be proportional to the number of damaged cells.

Examples of enzymes often analyzed in the clinical laboratory include amylase, glutamic oxaloacetate transaminase (GOT), creatine phosphokinase (CPK), and glutamic pyruvate transaminase (GPT). Amylase is primarily found in the tissues of the pancreas and salivary glands. It would then be released into the blood stream during tissue damage to these organs, such as would occur during pancreatitis or mumps. GOT is found in tissues of the heart and liver. Therefore, increased serum GOT values are expected following a heart attack or during liver disease. CPK, found in heart and muscle tissue, is released during a heart attack or muscle disease such as muscular dystrophy. GPT is found primarily in the liver. An increase in serum GPT level may indicate liver disease.

GOT, CPK, and GPT are quite often performed together on coronary victims to ascertain the degree of heart damage. GPT is run to indicate any GOT concentrations that might be due to liver damage. CPK serves as a check on the results.

Let's observe the enzyme amylase more specifically. Amylase is a hydrolytic enzyme that uses starch or glycogen as its substrate and produces maltose and dextrins, short chained polysaccharides. As mentioned earlier, amylase is produced by both the salivary glands and the pancreas.

The activity factors for amylase are essentially the same as for enzymes in general: temperature, pH, activators, inhibitors, time and concentrations. More specifically the optimum temperature range is from 50 to 55° C, the optimum pH range is between 6.9 and 7.2. Amylase needs either Ca^{++} or Cl^{-} ions present to be activated. Inhibitors include acids which denature the enzyme. (Amylase secreted in the mouth is denatured in the stomach and is replenished in the neutral to basic intestine by the pancreas.) Anticoagulants that bind up Ca^{++} remove all the Ca^{++} from solution, thus inactivating the enzyme. Other general inhibitors include organic solvents, detergents, and heavy metals. Other variables for activity include time and substrate concentration.

Amylase activity is determined by either measuring products formed or measuring remaining substrate. Three methods are employed for determining amylase activity; these are saccharogenic, amyloclastic, and iodometric methods. The saccharogenic method measures the amount of product formed. The amyloclastic method measures the amount of time it takes for the reaction to proceed to the metachromic point, the point where there is a distinct color change, indicating a depletion of substrate. The iodometric method is a color reduction test, measuring the remaining substrate. Iodine is added to the reaction after it has been allowed to proceed for a specified length of time. The resulting color production is indirectly proportional to the amount of amylase activity.

Normal values for amylase activity are 0.06-0.34 ImU/ml of serum or 60-160 Somogyi units. Decreased values are of little significance. Increased values indicate acute pancreatitis, obstruction of the pancreatic duct, or other diseases affecting these glands.

During this period of instruction, we have examined the chemical nature of enzymes, the need for cofactors, how enzymes function, the naming of enzymes, factors influencing enzyme activity, the measurement of enzyme activity, and the use of enzymes as a diagnostic tool. We have also taken a brief look at amylase as an example enzyme.

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IM 08-280-265
084

Enzyme determinations are requested with increased frequency by the physician. It is essential, then, for you as medical laboratory specialists to be familiar with the principles and pitfalls of enzymatic tests, so as to be able to perform these tests with precision and accuracy.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-270
LABORATORY SCIENCES DIVISION 068
CHEMISTRY BRANCH

Laboratory Instrumentation (4 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Procedures (Basic)

PLACE: Bldg. 2841, Rm. 2401

REFERENCES:

General Operations Instruction Manual for Basic AutoAnalyzer Modules. Tarrytown: Technicon Instruments Corp. 1966.
Tietz, N. E., ed. Fundamentals of Clinical Chemistry. Philadelphia: W. B. Saunders Company, 1976, pp 125-166, 188-198, 286-290, 305-315.
Davidsohn, I. and Henry, J. B., ed. Todd-Sanford Clinical Diagnosis by Laboratory Data. 15th ed. Philadelphia: W. B. Saunders Company, 1974. pp 502-508, 512-513, 547-560, 702-736, 782-785.
Lee, Leslie W., ed. Elementary Principles of Laboratory Instruments. 3rd ed. Saint Louis: C. V. Mosby Company, 1974, pp 99-111, 118-122, 137-156, 173-197, 209-211, 243-246.

STUDY ASSIGNMENT: After class, review lecture notes.

STUDENT UNIFORM AND EQUIPMENT: Laboratory notebook.

TOOLS, EQUIPMENT, AND MATERIALS: M-08-280-270-1

PERSONNEL: One instructor (MOS 92B4H), 1st hour; eight instructors (MOS 92B4H), 2nd thru 4th hours.

INSTRUCTIONAL AIDS: Instruments in/and Room 2401; chalkboard with chalk and eraser; overhead projector; 35mm slide projector, and screen.

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: L (1st and 2nd hours), PE (3rd and 4th hours)

FIRST HOUR

Note: Issue M 08-280-270-1.

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*This lesson plan supersedes LP 08-280-270, 084.

I. INTRODUCTION (5 min)

A. Opening Statement: As we have seen in the previous hours of instruction, the clinical chemistry section is an involved, in depth study center for testing abnormalities which can affect any organ of the body. To perform this broad scope of tests, using manual procedures, is time consuming and costly. To cope with this ever increasing work load, the field of clinical chemistry has turned to the use of automation.

B. Objectives

1. List the four advantages of using automated procedures in the clinical laboratory.

2. Given a list, select the basic types of separation techniques.

3. Select the two types of multitest analyzer techniques.

4. Select the six basic modules of the AutoAnalyzer and the major function(s) of each module.

C. Class Procedure and Lesson Tie-in: Lecture to be followed by PE for familiarization with the instruments presented in lecture.

II. EXPLANATION (80 min)

A. Advantage of Automation

1. Increases rate of analysis

2. Frees technician for other work

3. Reduces overall cost of analysis

4. Provides greater precision and accuracy

QUESTION: Define precision and accuracy.

ANSWER: Precision is the ability to reproduce one's results, whereas accuracy is the ability to reach or attain the correct value.

NOTE: Use T 08-280-270-1 and T 08-280-270-2 to develop Single Test Principles (see Annex A).

B. Single Test Techniques

1. Atomic absorption

a. Opposite of emission spectroscopy

QUESTION: What is the definition of emission spectroscopy?

ANSWER: Measuring the intensity of emitted light produced by a burning flame at characteristic wavelengths.

b. Sample atomized

c. Ground state atoms absorb light of a specific wavelength

d. Used to measure trace metals: Ca, Mg, and Zn

2. Fluorometry

a. Measures fluorescent emission

b. Common use

(1) Ca

(2) Cholesterol

(3) Porphyrins

3. Osmometry

a. Measures particle concentration in relation to colligative properties.

b. Approaches

(1) Freezing point depression

(2) Vapor pressure

(3) Dew point

(4) Boiling point

4. Radioimmuno assay

a. Measures organic substances by use of radioactively labeled antigen/antibody reaction

b. Used mainly for hormones and vitamins

5. Electrodes

a. Measures changes in electrical potential

b. Electrodes allow selective passage of ions from a solution

c. Ions passing into electrode will change the electrical potential

d. Types

(1) Ion specific

(2) Liquid membrane

6. Separation techniques

a. Chromatography

(1) Separates or purifies the components of a mixture of closely related compounds by their physical properties

(2) Mobile and stationary phases

(a) Liquid/liquid

(b) Gas/liquid

(c) Liquid/solid

(d) Gas/solid

(3) Types - paper, thin layer, gas

(4) Uses

Drugs, alcohols, and identification of bacteria by their waste products

b. Electrophoresis

(1) Separates or purifies the components of a mixture of closely related compounds by their electrical properties

(2) Principle: components of a mixture migrate differently through an electrical field

(3) Uses

(a) Isoenzymes

(b) Hemoglobins

(c) Proteins

C. Multitest Analyzers

1. Discrete

a. Performs one test in one tube

b. Types

(1) True discrete analyzer

(a) Dupont ACA

Samples are pipetted into individual test packets behind each other, mixed, read, and recorded

(b) Hycel series

Sample added to 10, 16, or 30 tubes, reagents added at appropriate time, results read with spectrophotometer, and printed out.

(2) Batch analyzer

Performs one test at a time on several samples

(a) Gilford 3500

(b) Several others

(3) Centrifugal analyzer

Reagents and samples mixed by centrifugal force and read by a computer as the cuvet spins past

(a) Centrifuchem

(b) Rotochem

(c) GEMSAC

2. Continuous flow analysis

All specimens are processed through the same tubing with a continuous input of reagents and samples.

NOTE: Use S 08-280-270-1 thru 40 on AA modules (see Annex B)

D. AutoAnalyzer Modules

1. Sampler

a. Functions

(1) Holds sample

(2) Controls rate of analysis

b. Components

Sampler, specimen tray, and cam

2. Proportioning pump with manifold

a. Functions

(1) Propels fluid stream

(2) Combines air, sample, and reagents

b. Components

(1) Roller pump

(2) Manifold

NOTE: The proportioning pump of an AutoAnalyzer is often referred to as the heart of the system.

3. Dialyzer

a. Function - dialysis

(1) Definition: Dialysis is the selective passage of ions or molecules across a semi-permeable membrane into an area of lesser concentration.

  - (2) Purpose: Separates the constituent to be measured from the interfering substances, such as proteins.

- b. Components
  - (1) Water bath (AAI only)
  - (2) Coils
  - (3) Membrane with press plates

4. Heating bath
   - a. Function

     Provides heat as a catalyst for chemical reactions
   - b. Components
     - (1) Bath - 37 or 95 degrees centigrade
     - (2) Coils - 40 feet long

5. Colorimeter
   - a. Functions
     - (1) Detect changes of color intensity
     - (2) Change light energy to electrical energy
   - b. Components
     - (1) Filters and apertures
     - (2) Flow cell
     - (3) Photocell

QUESTION: What module is often referred to as the heart of the AutoAnalyzer system?

ANSWER: The proportioning pump.

6. Recorder
   - a. Functions
     - (1) Changes electrical energy to mechanical energy
     - (2) Provides a written report of absorbancies

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b. Components

Recorder with chart paper and curve charts

7. Other modules

   a. Digestor column

   b. Fluorometer

   c. Flame photometer

   d. Digital calculator/printer

E. Question from Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Advantages of automation

2. Single test techniques

3. Multitest analyzers

4. AutoAnalyzer modules

B. Closing Statement: As you have now seen, the clinical chemistry section of the laboratory is forever expanding. This expansion includes both the number and type of tests being requested. Only with the application of automation can the laboratory assure the physician of rapid and accurate results.

FOURTH HOUR

IV. APPLICATION (80 min)

A. Instructors' Activities

1. Set up and operate AutoAnalyzer for simulated determination

2. Walk-through dicussion over instruments in Room 2401

3. Supervise students' activities

B. Students' Activities

1. Observe operation of the AutoAnalyzer

2. Observe operation of instruments covered in lecture

3. Clean up area and equipment as necessary

V. CRITIQUE (10 min)

A. Questions from Students

B. Closing Statement: With a good background in manual procedures, and the obvious need to learn, the technician can later move into the field of automation with confidence.

ANNEX B
INSTRUCTIONAL AIDS

Slides

S 08-280-270-1	ACA
S 08-280-270-2	Front view of ACA
S 08-280-270-3	AA modules
S 08-280-270-4	Sampler
S 08-280-270-5	Sampler with tray and cams
S 08-280-270-6	Functions
S 08-280-270-7	Holds sample, controls rate of analysis
S 08-280-270-8	Proportioning pump with manifold
S 08-280-270-9	Proportioning pump with manifold disassembled
S 08-280-270-10	Proportioning pump with manifold assembled
S 08-280-270-11	Principle of plates and rollers
S 08-280-270-12	Functions
S 08-280-270-13	Propel fluid stream, combine air, sample and reagent
S 08-280-270-14	Dialyzer
S 08-280-270-15	Dialyzer disassembled
S 08-280-270-16	Dialyzer assembled
S 08-280-270-17	Dialyzer membrane plate press open
S 08-280-270-18	Dialyzer membrane
S 08-280-270-19	Functions
S 08-280-270-20	Dialysis, separation
S 08-280-270-21	Heating bath
S 08-280-270-22	Heating bath and 40 foot coil
S 08-280-270-23	Mixing coil

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S 08-280-270-24	Functions
S 08-280-270-25	Provide heat to catalyze chemical reactions
S 08-280-270-26	Colorimeter
S 08-280-270-27	Colorimeter with flow cell exposed
S 08-280-270-28	Filters and apertures
S 08-280-270-29	Detect changes in color intensity, change light energy to electrical energy
S 08-280-270-30	Recorder
S 08-280-270-31	Recorder with chart exposed
S 08-280-270-32	Recorder front view
S 08-280-270-33	Change electrical energy to mechanical energy. Written record of Abs.
S 08-280-270-34	Complete system (6 basic modules)
S 08-280-270-35	Other modules: Digestor column, fluorometer, flame photometer and digital calculator/printer
S 08-280-270-36	AA II
S 08-280-270-37	SMA
S 08-280-270-38	SMA
S 08-280-270-39	SMAC
S 08-280-270-40	Result sheet (SMAC)

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *IM 08-280-270
LABORATORY SCIENCES DIVISION 068
CHEMISTRY BRANCH

Laboratory Instrumentation

As we have seen in the previous hours of instruction, the clinical chemistry section is an involved indepth study center for testing abnormalities which can affect any organ of the body. To perform this broad scope of tests, using manual procedures, is time consuming and costly. Thus, to cope with this ever increasing work load, the field of clinical chemistry has turned to the use of automation.

The four advantages seen in the use of automation are an increased rate of analysis, technicians freed for other work, the overall cost of analysis reduced, and greater precision and accuracy provided.

Generally, automated equipment used in clinical chemistry can be classified as single test or multi-test systems. Included in these systems are a multitude of procedures which can serve the technician, physician, and patient.

We have previously used a form of automation, when performing sodium and potassium determinations, using the principle of emission spectroscopy. Remember that emission spectroscopy is the measurement of emitted light when ions are burned in a flame. From this method of measurement has evolved the principle of atomic absorption. This method employs the principle that un-ionized atoms will absorb light of the wavelength that it would emit if emission flame spectroscopy were used. With this instrument we can measure trace metals such as calcium, magnesium, and zinc.

Another form of emission measurement is used in fluorometry. With this technique fluorescent emission is utilized to measure such things as calcium, cholesterol, and porphyrins.

Osmometry is another useful technique employed in the laboratory today. This method measures the physiochemical properties of solutions. Those properties include osmotic pressure, vapor pressure, boiling point, and freezing point.

Radioimmuno assay, which uses a radioactive labeled antibody/antigen reaction to measure organic substances, has also come into existence of late with the increasing consideration given to hormone and vitamin levels. This technique is increasing in importance and use.

We have also seen previously that electrodes can serve an extremely useful role in clinical chemistry to measure changes in electrical potential.

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*This instructor's manuscript supersedes IM 08-280-270, 084.

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These electrodes allow only selected passage of ions from the solution being measured which changes the electrical potential within the electrode. The most common types of electrodes are the ion specific and liquid membrane electrodes.

We have now seen how to measure one substance at a time using automation; however, if the physician requires identification of any one or many components of a given specimen, that are extremely similar, the above mentioned procedures will prove inadequate. For this purpose the specimen must be separated; here the two basic separation techniques are chromatography and electrophoresis. Chromotography separates or purifies the components of a mixture of closely related compounds by their physical properties. With this type of technique it is possible to separate alcohols, drugs, and even identify bacteria by their waste products. This principle employs a mobile and/or stationary phase and either a liquid-liquid, gas-liquid, liquid-solid, or gas-solid column to aid in separation of the components. These substances are then measured against a known solution standard and the two are compared. Electrophoresis allows us to separate closely related compounds by their electrical properties. Using this method of separation the specimen is placed on an agar-like plate and then placed in an electrical field to allow migration according to the constituents' electrical properties. This method is commonly used to separate isoenzymes, proteins, and hemoglobins.

Multi-test instruments can be classified into two groups: discrete, and continous flow. The discrete analyzer performs many tests at the same time without manual pipetting. Among these discrete analyzers are the Dupont ACA and the Hycel series. These analyzers perform tests with the specimen being added into individual test reagent packets, one behind the other as with the Dupont ACA or by addition of the specimen in each programmed tube on a conveyer, where reagents are then added at the proper time as with the Hycel series. All tests are carried and mixed on a conveyor to the readout device which records and prints out the results. These two instruments are also known as "true discrete" analyzers.

Batch analyzers are also discrete analyzers; however, they are limited in that they can only run one type of test at a time, but run several samples in a short time. Common batch analyzers seen in the field are the Gilford 3500 and enzyme analyzers.

There are also instruments which mix the sample and reagents by centrifugation and use a computer to read the cuvet as it spins by. These are known as centrifugal analyzers. Those most often seen are the Centrifuchem, Rotochem, and GEMSAC.

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Let us now look at the second type of multi-test instruments known as continuous flow analyzers. Using this type of analysis all specimens are processed through the same tubing with a continuous input of reagents and samples. The AutoAnalyzer falls into this category and is still the most common instrument in the chemistry laboratory. This instrument is composed of six primary modules. The first is the sampler, which is responsible for holding the specimen, aspiration of sample into system, and controlling the rate of analysis. The sampler also includes the specimen tray and cam. The second module is the proportioning pump which propels the fluid stream and combines the sample and reagents. The proportioning pump also includes the manifold, tubing, and fittings. It should also be noted that the proportioning pump is referred to as the heart of the system. The third module is the dialyzer which is responsible for purification of the specimen by dialysis. This is accomplished with a porous membrane that separates the waste material from the part of the specimen being tested. The dialyzer also consists of a water bath and small mixing coils. The heating bath is the fourth module and is responsible for creating heat to act as a catalyst for chemical reactions. This bath can be either 37° centigrade or 95° centigrade. There is also a 40 foot glass coil inside the heating bath for mixing and timing purposes. After being mixed and heated, the specimen will travel through the colorimeter which serves as a spectrophotometer, and is the fifth module. The colorimeter, like the spectrophotometer, detects changes in color intensity and converts light energy into electrical energy. Included with the colorimeter are a set of filters, apertures, and a flow cell, which serves as a cuvet. The sixth and final module is the recorder, and like the readout device of the spectrophotometer, serves the same purpose. The recorder changes electrical energy to mechanical energy and will also give a written report of absorbance. These are the six basic modules found in most AutoAnalyzers; however, there are other modules used for more specific tests. These include a digestor column, fluorometer, flame photometer, and digital printer.

As you have now seen, the clinical chemistry section of the laboratory is forever expanding. This expansion includes the number and type of tests being requested. Only with the application of automation can the laboratory assure the physician of rapid and accurate results.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY M 08-280-270-1
LABORATORY SCIENCES DIVISION 127
CHEMISTRY BRANCH

Laboratory Instrumentation

I. INTRODUCTION

A. Objectives

1. List the four advantages of using automated procedures in the clinical laboratory.

2. Given a list, select the basic types of separation techniques.

3. Select the two types of Multitest Analyzer Techniques.

4. Select the six basic modules of the autoanalyzer and the major function(s) of each module.

B. Class Procedure and Lesson Tie-in

Lecture to be followed by practical exercise for familiarization with the instruments presented in lecture.

II. EXPLANATION

A. Advantages of Automation

1.

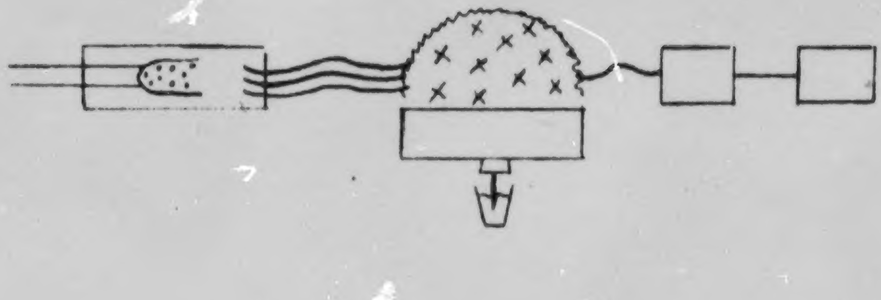
2.

3.

4.

B. Single Test Techniques

1.



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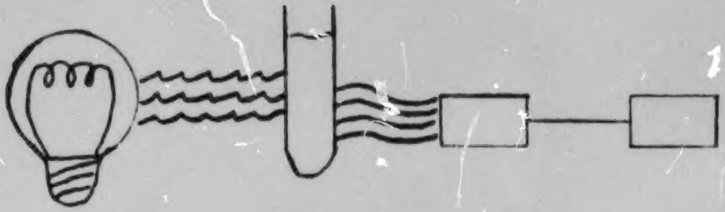
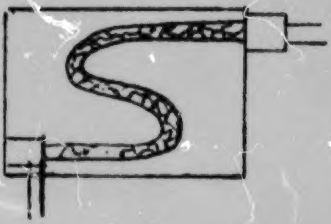


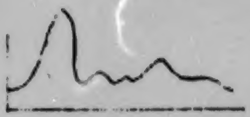
3.

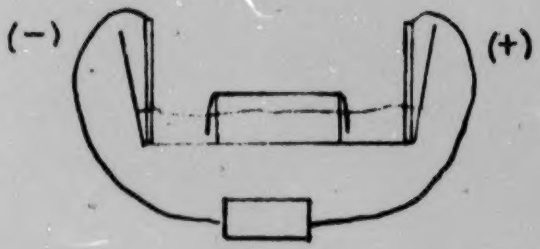
4.

5.

6. Separation techniques







C. Multitest Analyzers

1.

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2.

D. AutoAnalyzer Modules

1.

2.

879

3.

4.

5.

6.

7.

SSG Tony G. Hedges

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-275-1
LABORATORY SCIENCES DIVISION 067
CHEMISTRY BRANCH

311-92B10 Chemistry Review

A. Spectrophotometry

1. Select the six main parts of the spectrophotometer and the main function of each part.
2. Based on Beer's Law, calculate sample concentrations and select the two factors proportional to absorbance.
3. Select the definition of selective absorption.
4. Given a standard curve and unknown absorbances, determine unknown concentrations.
5. Determine the absorbance of unknown samples by using a spectrophotometer, to include calibrating, zeroing, and blanking of the instrument.

B. Volumetric Glassware

1. Select the most common use of volumetric flasks in clinical chemistry.
2. Select the two types of calibration used on laboratory glassware.
3. Select the types of pipets, the use, type of calibration, and the method of delivery for each.
4. With practice, correctly use volumetric glassware to prepare reagents and perform laboratory determinations.

C. Quality Control

1. Recognize the definitions of quality control, standard, control, reagent blank, accuracy, precision, and mean.
2. Given a quality control graph, select the acceptable limits of variation from the mean of control values, and recognize which of the following abnormal conditions has occurred: out-of-control values, shift, or trend.
3. Select three possible sources of error and items to be monitored in the clinical laboratory.
4. With practice, plot control values on a quality control chart.
5. With practice, use controls in laboratory procedures for monitoring the accuracy of results.

D. Carbohydrates

1. Select the elements found in all carbohydrates.
2. Select three classes of carbohydrates based on the number of simple sugar units in each.
3. Select the name of the reaction of glucose breakdown in the body, the end products, and the forms and the primary locations of excess glucose storage.
4. Recognize the name of the major hormone controlling blood glucose levels, and its effect on those blood glucose levels, to include the names of patient conditions.
5. Select the preservative of choice and the alternate available method for drawing a glucose specimen.
6. Recognize two types of specimens based on time of collection.
7. Select the two body fluids tested in the Glucose Tolerance Test.
8. Recognize three glucose test methods, and the limitations of each.
9. Select the normal values for serum and CSF glucose concentrations.

E. Protein and Kidney Function

1. Select the four chemical elements found in all proteins.
2. Select the function of the peptide bond in protein molecules.
3. Recognize the four major functions of proteins in the body.
4. Select the two major classifications of proteins and the components of each.
5. Recognize the toxic and relatively nontoxic end products of protein breakdown and the respective organs responsible for the creation and removal of urea.
6. Select the two major functions of the kidney.
7. Select the three structural units of the nephron and the major function of each unit.
8. Recognize the range of normal values for serum total protein and blood urea nitrogen.

9. Recognize the clinical significance of elevated or decreased blood urea nitrogen values.

10. With practice, satisfactorily perform blood urea nitrogen determinations by the diacetyl monoxime thiosemicarbazide method.

F. Chloridometer

1. Select the normal value ranges for serum and CSF chloride concentrations.

2. With practice, satisfactorily quantitate serum and CSF chloride concentrations using the chloridometer.

G. Electrolytes

1. Select the definition of electrolytes, cation, and anion.

2. Recognize the three compartments of body fluid, and select the percent composition of each compartment as it relates to total body weight.

3. Select the chief anion and cation of each of the three fluid compartments.

4. Recognize the effect of hypertonic, hypotonic, and isotonic solutions on a cell.

H. pH and Blood Gases

1. Select the relationship between pH and hydrogen ion concentration.

2. Select the pH ranges for acidic, basic, and neutral solutions.

3. Recognize the main function and the components of a buffer system.

4. Recognize the description of the alkali reserve of the body.

5. Select the enzyme involved in CO_2 transportation, the name of the phenomena in which HCO_3^- and Cl^- exchange places, and the major form and location in which CO_2 is transported.

6. Select the normal values for blood pH, HCO_3- concentration, PO_2, and the ratio of the concentrations of HCO_3- and H_2CO_3.

7. Recognize the direction of the change in the HCO_3-/H_2CO_3 ratio for each of acidosis and alkalosis.

8. Recognize the two organs responsible for regulation of body pH.

9. With practice, satisfactorily perform manometric CO_2 determinations.

I. IL Flamephotometer

1. Select the principle of flame photometry.

2. With practice, satisfactorily perform sodium and potassium determinations.

J. Bilirubin

1. Recognize the four basic functions of the liver.

2. Select the substance which breaks down to bilirubin, and where in the body this breakdown occurs.

3. Select the two basic forms of bilirubin, where they are formed, their common synonyms, and select the normal serum values of each.

4. Select the four types of jaundice; a related cause and type of bilirubin that is increased for each type.

5. Select the subsequent organs involved in bilirubin excretion, and their respective roles in that excretion.

6. With practice, satisfactorily perform the Evelyn-Malloy serum bilirubin determinations.

K. Enzymes

1. Recognize the definitions of enzyme, substrate, product, and enzyme activity.

2. Select the six factors which influence enzyme activity.

3. Recognize the three approaches to measuring enzyme activity.

4. Select the quality of an enzyme which makes it valuable as a diagnostic tool.

5. Select the two types of cofactors.

L. Autoanalyzer

1. Select the six basic modules of the Autoanalyzer and the major functions of each module.

SSG John Blandamer

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY M 08-280-275-2
LABORATORY SCIENCES DIVISION 077
CHEMISTRY BRANCH

Course Review Questions

A. Spectrophotometry

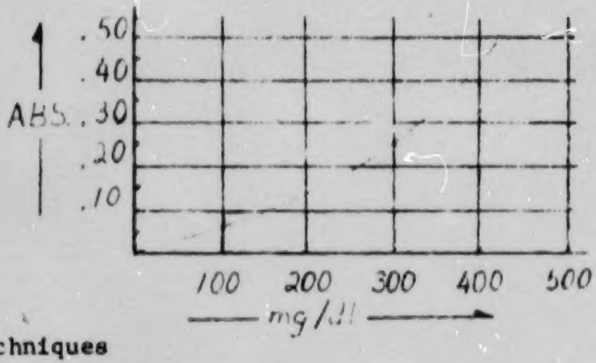
1. List the six main parts of the spectrophotometer and the main function of each part.

PART	FUNCTION

2. The two factors which are proportional to absorbance according to Beer's Law are ______________________ and ____________.

3. If a 200 mg/dl glucose standard has an absorbance of .39 what is the concentration of an unknown which has an absorbance of .23?

4. Using the standard curve below what is the concentration of an unknown having an absorbance of .25?



B. Analytical Techniques

1. The most common use of volumetric flasks in the clinical laboratory is ____.

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*This mimeo supersedes M 08-280-275-2, 022.

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2. The two types of calibration used for laboratory glassware are ______________ and ____________.

3. Complete the table below for each of the four types of pipets.

TYPE	USE	CALIBRATION	METHOD OF DELIVERY

4. Define the following terms:

   a. Quality control

   b. Standard

   c. Control

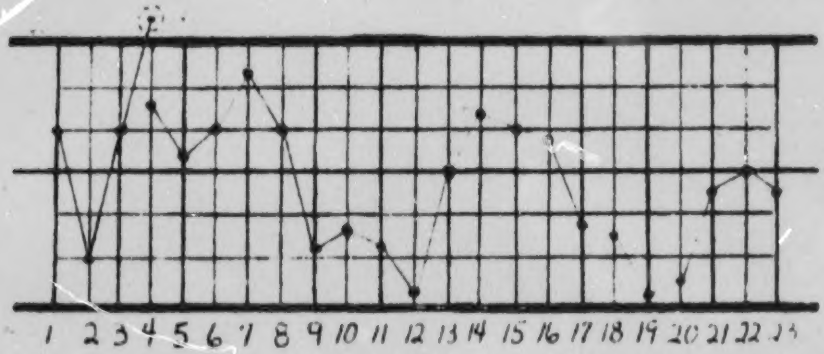
   d. Reagent blank

   e. Accuracy

   f. Precision

   g. Mean

5. On the quality control graph below the acceptable limits of variation from the mean are indicated by the dark lines above and below the mean. These acceptable limits are ____________.



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6. Based on the graph above identify the days on which the following abnormal conditions occurred: out-of-control __________ shift ______________, trend ________________.

7. The three sources of error and items to be monitored in the laboratory are ______________, ____________, and ______________.

C. Carbohydrates

1. All carbohydrates contain what three elements?

______________________________ ______________________ ______________

2. Match the class of carbohydrate with the number of simple sugar units in each.

____ a. Monosaccharide 1. 3 or more sugar units

____ b. Disaccharide 2. 2 sugar units

____ c. Polysaccharide 3. 1 sugar unit

3. The reaction whereby glucose is broken down in the body is __________________. The end products of this reaction are ____________ __________, ____________, and ________________.

4. Excess glucose may be stored as ______________ primarily in the ____________ and ____________ or as _____ in __________ ___________.

5. The major hormone controlling glucose levels in the blood is ______________. Increased production of this hormone, a condition known as __________________ will result in (hypo/hyper) glycemia. Decreased production of this hormone, a condition known as ________________________ will result in (hypo/hyper) glycemia.

6. The preservative of choice for blood glucose specimens is ______________ ____________. An alternate method available for drawing blood glucoses is the ____________ ____________.

7. The two types of specimens based on time of collection are ____________ and ______________________.

8. When performing the glucose tolerance test the two body fluids tested are _______________ and _______________.

9. List the three glucose test methods and a limitation of each.

METHOD	LIMITATION
____________	____________________
____________	____________________
____________	____________________

10. The normal range for serum glucose is ________ to ________ mg/dl.
The normal range for CSF glucose is ________ to ________ mg/dl.

D. Protein and Kidney Function

1. The four elements found in all proteins are ____________, ____________, ____________, and ____________.

2. The type of bond which links amino acids together to form protein molecules is the ______________ bond.

3. The four major functions of proteins in the body are __________, ______________, ____________, and secondary ____________ ___________.

4. Proteins may be grouped into two major classifications. Simple proteins which contain ____________ ______________ only and ______________ proteins containing __________ ____________ and a nonprotein portion called a ______________ __________.

5. The toxic end product of protein breakdown, __________, is converted to a relatively non-toxic end product ___________ in the _________. This relatively non-toxic product is then removed from the system by the _____________.

6. The two major functions of the kidney are _____________ and _________________.

7. The basic functional unit of the kidney is the nephron. In the blanks below list the three structural units of the nephron and the major function of each.

UNIT	FUNCTION
____________________	____________________
____________________	____________________
____________________	____________________

The normal range for serum total protein is ________ to ______ g/dl.

The normal range for blood urea nitrogen is _____ to _____ mg/dl.

10 Elevated BUN values (greater than 25 mg/dl) would be an indication of ______________________________, ___________________________, or ______________________________.

11. Decreased values (less than 5 mg/dl) are of what clinical significance?

E. Chloridometer

1. The normal values for serum chloride are ________ to ______ mEq/L.

2. The normal values for cerebrospinal fluid (CSF) chloride are __________ to __________ mEq/L.

F. Electrolytes

1. Define the following terms:

   a. Electrolyte

   b. Cation

   c. Anion

2. Complete the chart below for the two compartments of body fluid.

COMPARTMENT	CHIEF CATION	CHIEF ANION
Intracellular		HPO_4
Extracellular		

3. Describe the effect each of these solution tonicities would have on a cell:

   a. Hypertonic solution:

   b. Hypontonic solution:

   c. Isotonic solution:

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G. pH and Blood Gases

1. pH and hydrogen ion concentration are inversely related. This means that a change in pH from 14 to 3 is caused by an (increase/decrease) in hydrogen ion concentration.

2. The pH ranges for an acidic solution are ______ to _____, for a basic solution ______ to _______, and a neutral solution has a pH of _________.

3. The main function of a buffer solution is to ______________________ ________________. A buffer system is composed of a ____________ ____________ with its salt or a _______ ________ with its salt.

4. Bicarbonate (HCO_3^-) ions in the plasma constitute the __________ ___________ of the body.

5. The enzyme ____________________ __________________ is involved in the transportation of CO_2 in the body and is contained in the RBC. The phenomena in which bicarbonate ions and chloride ions exchange places is known as the ______________ _________________.

6. Most of the carbon dioxide in the body is transported in the form of _________________ in the ________________.

7. The normal range for blood pH is __________ to __________. The normal range for HCO_3^- is ______ to _______ mEq/L. The normal range for arterial pO_2 is _________ to ________ mm Hg. What is the normal ratio of the concentrations of HCO_3^- and H_2CO_3 in the body? _________/_________

8. An increase in the ratio of HCO_3^-/H_2CO_3 will result in a condition known as ________________________. A decrease in this same ratio will result in a condition known as ___________________________.

9. The two organs responsible for regulation of body pH are the ___________ and the ______________.

H. IL Flame Photometer

1. The principle of the IL flame photometer is _________________ ____________________.

I. Bilirubin

1. The four basic functions of the liver are ______________________, ________________, ______________, and ______________________.

2. Bilirubin is formed as the result of ________________ breakdown. This breakdown occurs in the __________________ ______________.

3. The two basic forms of bilirubin are __________ and ____________ bilirubin. Complete the chart below about each of these two forms.

Forms	Synonyms	Normal Values	Where Formed
__________	Conjugated	____ to ____ mg/dl	_____________
	_____________		
__________	Unconjugated	_____ to ____ mg/dl	_____________
	_____________		
	_____________		
	_____________		

4. There are four types of jaundice. For each type list its cause and the type of bilirubin increased.

TYPE	CAUSE	TYPE BILI. INCR.
_____________	___________________	________________________
_____________	___________________	________________________
_____________	___________________	________________________
_____________	___________________	________________________

5. Other organs involved in bilirubin excretion are the ________ ______________ which stores bile prior to its excretion into the ___________ _______________. Most of the bilirubin is then excreted by the large intestine; however some is reabsorbed into the bloodstream and excreted by the _____________________.

J. Enzymes

1. Define the following terms:

a. Enzyme -

b. Substrate -

c. Product -

d. Enzyme activity -

2. Six factors which influence enzyme activity are ______________, __________, ____________________, ____________________ ____________, ______________.

3. What are the three approaches to measuring enzyme activity?

____

____

____

4. The quality of an enzyme wich makes it valuable as a diagnostic tool is the fact that enzymes are ____________ or ____________ specific.

5. Two types of cofactors are ____________and ______________.

K. Autoanalyzer

1. List the six basic modules of the autoanalyzer and the function of each module.

Module	Function

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-280
LABORATORY SCIENCES DIVISION 084
CHEMISTRY BRANCH

Practical Examination and Reteaching Session (9 hours)

COURSES PRESENTED TO: 311-92B20, Medical Laboratory Procedures (Basic)

PLACE: Classroom/laboratory

REFERENCES: All previous references

STUDY ASSIGNMENT: Before class, review methods for determining concentrations of bicarbonate, sodium, potassium, bilirubin, and amylase in body fluids

STUDENT UNIFORM AND EQUIPMENT: Slide rule and laboratory notebook

TOOLS, EQUIPMENT, AND MATERIALS: Specimens and reagents for the determination of bicarbonate, sodium, potassium, bilirubin, and amylase in body fluids, issued at the beginning of the first hour

PERSONNEL: Three (3) instructors (MOS 92B4H) 1st thru 8th hours; one (1) instructor (MOS 92B4H) 9th hour

INSTRUCTIONAL AIDS: Chalk, chalkboard, and eraser

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: GPE (1st thru 8th hours), C (9th hour)

FIRST THROUGH EIGHTH HOURS

I. INTRODUCTION (5 min)

A. Opening Statement: In clinical chemistry, the mastery of performing basic procedures is of utmost importance. A medical laboratory specialist may have vast knowledge of chemical reactions occuring during the test procedures; however, his inability to satisfactorily perform routine determinations may directly affect the patient's welfare.

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*This lesson plan supersedes LP 08-280-330, 081.

B. Objectives

1. Satisfactorily perform determinations of bicarbonate, sodium, potassium, bilirubin, and amylase concentrations in body fluids using methods learned during previous exercises.

2. Accurately calculate and report results of routine determinations without supervision.

C. Class Procedure and Lesson Tie-in

Relates to hours previously presented on the determination of body fluid constituents.

II. APPLICATION (355 min)

A. Instructors' Activities

1. Provide specimens and reagents for the determination of bicarbonate, sodium, potassium, bilirubin, and amylase concentrations in body fluids.

2. Supervise students' activities.

3. Collect and grade students' report sheets,

B. Students' Activities

1. Satisfactorily perform determinations of bicarbonate, sodium, potassium, bilirubin, and amylase concentrations in specimens presented by the prescribed methods.

2. Maintain proper records.

    a. Laboratory notebook

    b. Quality control chart

3. Accurately calculate test results.

4. Report test results to instructors.

5. Clean area and equipment as necessary.

NINTH HOUR

III. CRITIQUE (45 min)

A. Questions From Students

B. Review of Main Points

1. Errors in technique

2. Errors in calculating and recording test results

3. Safety precautions

C. Closing Statement: The ability of a medical laboratory specialist to satisfactorily perform routine procedures is of utmost importance to the physician in treating patients. The physician most often relies on accurate laboratory test results as an aid to diagnosis of the patients' condition. As medical laboratory specialists, it behooves you to do your best to provide him with rapid and accurate test values.

SECTION IV - ANNEXES

ANNEX E

BASIC PROCEDURES IN MEDICAL BACTERIOLOGY

OBJECTIVE: Given necessary materials and bacterial cultures, perform, interpret and report Gram stains, streak media for isolation, report morphological characteristics of bacterial growth, inoculate and interpret biochemical tests, identify bacteria based on their morphological characteristics and reactions; prepare bacteriological media; discuss and or perform sterilization/decontamination procedures.

Subject and LP No	Lesson Objective	References
Introduction to Bacteriology 08-280-401	Given a list of descriptions of bacterial cell structures, identify the structures and/or select from a given list, the functions that they serve.	TM 8-227-5, chap 1, 17; Lennette, *et al.*, chap 1.
Sterilization and Decontamination 08-280-402	Given common laboratory situations involving sterilization/decontamination of media, equipment, and work areas, select the most correct means of accomplishing each. Given a list of various thermolabile and thermostable solids and liquids, select the most correct method of sterilization/decontamination for each. Given a typical laboratory situation, select the correct sterilization/decontamination procedures for waste disposal, accident clean-up and work area preparation. Given a situation, select the correct use of the autoclave for routine sterilization/decontamination procedures.	TM 8-227-5, chap 4; Lennette, *et al.*, chap 1, 93; VT 472.
Media Preparation 08-280-403	Given a situation, select the correct steps in preparing bacterial media and the correct steps required to quality control that media. Given a list of the various types of media, select the correct type of media based	GR 09-280-456-1; Difco Lab; Lennette, *et al.*, chap 95; VT 646.

Subject and LP No	Lesson Objective	Referenc
	on agar concentration. Perform the calculations required to prepare media as demonstrated in class.	
Media Preparation Practical Exercise 08-280-404	Given all necessary equipment and instructions, correctly prepare bacterial media suitable for a practical exercise, as demonstrated in class.	All previou references.
Nutrition and Cultivation of Bacteria 08-280-405	Given a list of nutritional and environmental requirements of the medically important bacteria, select the requirements that are needed for bacterial growth. Given a growth curve, select the name of the various growth phases and select the phase best suited for biochemical evaluation of the growth in Clinical Bacteriology. Given a source of nutrition, select the pH (acid or alkaline) resulting from the bacterial utilization of that source. Given a list of oxygen requirements and bacterial growth patterns in those oxygen environments, select the correct oxygen requirement for those bacteria. Given a list of media contents, select the correct classification of that media based on those contents.	Bailey & Scott, chap 1; Lennette, et al., chap 6.
Inoculation, Streaking of Media, and Colony Morphology 08-280-406	Given a clinical bacteriology situation, select the correct method of inoculating tube and plate media using the loop, needle, and swab. Given a list, select the correct steps in the performance of the "four quadrant" streaking method. Given a clinical bacteriology situation, select the	GR 08-280-465-1; Bailey & Scott, chap 4; Lennette, et al., chap 6; VT 188.

Subject and LP No	Lesson Objective	References
	steps used for subculture or re-isolation of a bacterial growth. Given a description of a bacterial colony growing on any of the plated media taught in class, select the correct colony morphology for those bacteria.	
Inoculation Streaking of Media, and Colony Morphology Practical Exercise 08-280-407	Given bacterial specimens and all necessary equipment, inoculate media, streak for isolation, and report colony morphology, as demonstrated in class.	All previous references.
The Gram Stain and Microscope Use 08-280-408	Given a list of steps, select the steps in the proper sequence for performing a Gram stain. Given a description of a Gram stained specimen, select the correct Gram morphology to include reaction, shape and arrangement. Given a situation, select the proper technique for storage, handling and operation of the microscope.	Bailey & Scott, chap 2, 3; Lennette, *et al.*, chap 6, 96; VT 187.
Gram Stain Practical Exercise 08-280-409	Given bacterial specimens and all necessary materials, stain, interpret, and report Gram morphology of smears, as demonstrated in class.	All previous references.
Cocci Biochemical Testing Procedures 08-280-410	Given a description of biochemical test reactions, select the correct reporting terminology. Given a list of cocci biochemical principles, select the principle that applies to specific biochemical tests.	GR 09-280-456-1; Bailey & Scott, chap 39, 40, 41; Lennette, *et al.*, chap 95, 96; VT 163.

Subject and LP No	Lesson Objective	References
Cocci Biochemical Testing Practical Exercise 08-280-411	Given bacterial cultures and all necessary materials, perform, interpret, and report results of cocci biochemical tests, as taught in class.	All previous references.
Streptococcus Species 08-280-412	Given criteria used in the identification of the Streptococcus species, to include nutritional and environmental requirements, colony morphology, and biochemical test reactions; select criteria that are used in the identification of the Streptococcus species. Given a list of specimen sources and pathogenic processes, select the source and or process where the Streptococcus species may be a disease causing agent.	Bailey & Scott, chap 17; Lennette, et al., chap 8, 9.
The Throat Specimen 08-280-413	Given a situation involving the collection and processing of a throat specimen, select the correct steps used in the proper collection and processing of a throat specimen. Given a list of bacteria, select the bacteria most commonly found in a throat specimen. Given a list of reporting terminologies and various types of bacterial flora, select the correct reporting terminology for the various types of bacteria.	Bailey & Scott, chap 8; Lennette, et al., chap 6.
Staphylococcus Species 08-280-414	Given criteria used in the identification of the Staphylococcus species, to include nutritional and environmental requirements, colony morphology, and biochemical test reactions; select criteria that are used in the identification	Bailey & Scott, chap 16; Lennette, et al.,

Subject and LP No	Lesson Objective	References
	of the Staphylococcus species. Given a list of specimen sources and pathogenic processes, select the source and or process where the Staphylococcus species may be a disease causing agent.	
Neisseria Species 08-280-415	Given criteria used in the identification of the Neisseria species, to include nutritional and environmental requirements, growth characteristics, and biochemical test reactions; select criteria that are used in the identification of the Neisseria species. Given a list of specimen sources and pathogenic processes, select the source and or process where the Neisseria species may be a disease causing agent.	GR 09-280-465-1; Bailey & Scott, chap 19; Lennette, et al., chap 10, 11.
Throat Specimen Practical Exercise 08-280-416	Given bacterial specimens and all necessary materials, inoculate and streak media for isolation, interpret and report colony morphology, perform and report all tests necessary to identify Beta hemolytic Streptococci, as demonstrated in class.	All previous references.
Sterilization and Decontamination Practical Exercise 08-280-417	Utilize the autoclave, dishwasher, or other means of sterilization and cleaning to decontaminate and/or prepare materials for disposal or reuse, as demonstrated in class.	All previous references.
Sterilization Examination 08-280-418	Given a situation, state the proper sterilization and or decontamination methods or procedures for 90 percent of the situations given in the following categories; autoclave operation, waste	All previous references.

Subject and LP No	Lesson Objective	References
	disposal, accident decontamination, work area preparation, and thermolabile material sterilization.	
Gram Stain Examination 08-280-419	Given bacterial specimens, and all necessary materials, prepare, stain and interpret the smear, and report the correct Gram morphology for each organism given, according to the standards stated in the performance evaluation checklist.	All previous references.
Classification of Gram Negative Bacilli 08-280-420	Given a list of characteristics and identification methods of the family Enterobacteriaceae, nonenteric gram negative bacilli, and fastidious gram negative bacilli, select characteristics or identification methods used in the identification of those bacteria.	Bailey & Scott, chap 20, 21; Lennette, et al., chap 18, 23, 24.
Stool Specimen Practical Exercise 08-280-421	Given simulated stool specimens, and all necessary materials, inoculate media, interpret and report colony morphology and reactions of biochemical tests used in the identification of intestinal pathogens, as demonstrated in class.	All previous references.
Enteric Biochemical/ Flow Chart Examination 08-280-422	Given a bacteria identification flow chart and biochemical color reactions, identify the bacteria described in 90 percent of the situations.	All previous references.
Enteric Media 08-280-423	Given an enteric media, select the correct classification for that media. Given a description of bacterial growth of an enteric medium, select the correct reporting terminology for for that growth. From a	Bailey & Scott, chap 39; BBL; Difco Lab; Lennette, et al., chap 95.

Subject and LP No	Lesson Objective	References
	list of bacteria, select the bacteria that will grow and those that are inhibited by various enteric media.	
Triple Sugar Iron Agar (TSI) 08-280-424	Given a description of a properly inoculated and incubated TSI, select the correct reporting terminology. Given a list of pathogenic Enterobacteriaceae, select the TSI reaction(s) that may be demonstrated by those bacteria. From the material presented in lecture, select the proper inoculation, and incubation procedure for TSI.	Bailey & Scott, chap 39; BBL; Difco Lab; Lennette, et al., chap 95; VT 514.
Kirby-Bauer Susceptibility Testing 08-280-425	Given a list of media, select the one to be used for a Kirby-Bauer susceptibility test. Given a list of procedures, select the steps in preparing inoculum for a Kirby-Bauer susceptibility test. Given a list of procedures, select the steps in preparing and inoculating sensitivity plates for the Kirby-Bauer susceptibility test. Given a list of possible ways to interpret zone sizes, select the correct method.	GR 09-280-465-1; Bailey & Scott, chap 34; Lennette, et al., chap 44, 46; VT 163.
Enteric Biochemicals 08-280-426	Given a list of enteric biochemicals, select the correct method of inoculation and incubation. Given a description of properly inoculated and incubated enteric biochemicals, select the correct reporting terminology for those biochemicals. Given an enteric biochemical, select the biochemical test principal involved in its use.	GR 09-280-456-1; BBL; Difco Lab.

Subject and LP No	Lesson Objective	References
Cocci Crucial Examination 08-280-428	Given cocci biochemical test results, select the correct report for the biochemical test or the correct name for the bacteria described for 90 percent of the situations given.	All previous references.
The Bacterial Stool Specimen 08-280-429	From a list of bacteria, select five that are intestinal pathogens. Given a list of various media, select those to be inoculated for a stool culture. Given a situation, select the steps and/or procedures used to identify intestinal pathogens.	Bailey & Scott, chap 9; Lennette, et al., chap 6.
Aerobic - Anaerobic Practical Exercise 08-280-431	Given known bacterial specimens, inoculate and streak media for isolation, observe and interpret the oxygen requirements of each of the given bacteria, as demonstrated in class.	Bailey & Scott, chap 3, 5; Lennette, et al., chap 37, 38.
Introduction to Identification Schema 08-280-432	Given an identification schema, and biochemical test results, select the correct biochemical tests to use and the correct name of the bacteria described.	Bailey & Scott, chap 20; BBL; Difco Lab.
Mixed Organism Practical Exercise 08-280-433	Given necessary materials and simulated throat, stool, and cerebrospinal fluid specimens, select the proper media, correctly inoculate and streak agar plates, select proper incubation environment, perform appropriate tests and stains, correctly interpret and report results of the tests and identify the pathogens present, as demonstrated in class.	All previous references.

ubject and .P No	Lesson Objective	References
linical pecimens 8-280-434	Given a bacteriology specimen, select the proper method for collection, processing, and medium of choice. Given a bacteriology specimen, select bacteria that may be pathogenic in that specimen.	GR 09-280-465-1; Bailey & Scott, chap 6, 8, 10, 11, 13; Lennette, et al., chap 6.
he Cerebro- pinal Fluid pecimen 8-280-435	Given a cerebrospinal fluid specimen, select the correct procedure for collection and processing of that specimen. From a list of organisms, select those that can be pathogenic in spinal fluid. Given identifying characteristics, select the characteristics that are used in the identification of Haemophilus influenzae to include the use of chocolate agar.	Bailey & Scott, chap 12, 95; Lennette, et al., chap 6, 39.
dentification f Bacteria xamination 8-280-436	Given simulated spinal fluid specimens, perform initial gram stains and report the presence or absence of bacteria to 100% accuracy. Given simulated throat, stool, and cerebrospinal fluid specimens, correctly identify and report pathogens present by utilizing necessary material provided and specified identification charts, to an accuracy of 90 percent.	All previous references.
irby Bauer ractical xercise 8-280-437	Given bacterial cultures and necessary materials, perform, interpret and report results of the Kirby Bauer susceptibility test, as demonstrated in class.	All previous references.

SECTION IV - ANNEX E (Cont)

Subject and LP No	Lesson Objective	References
Cerebrospinal Fluid Specimen Practical Exercise 08-280-438	Given simulated cerebrospinal fluid specimens, concentrate, gram stain the sediment, inoculate media and streak for isolation, interpret and report colony morphology, perform and report all tests necessary to identify bacteria present in the specimens, as demonstrated in class.	All previous references.
Written Examination 08-280-439	Answer questions pertaining to previously taught material as expressed by the lesson plan objectives.	All previous references.
API Practical Exercise 08-280-440	Given bacterial specimens and all necessary materials, prepare, inoculate, and interpret an API test strip, as demonstrated in class.	API Brochure.

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SFC James A. Goode

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-401
LABORATORY SCIENCE DIVISION 038
MICROBIOLOGY BRANCH

Introduction to Bacteriology (1 hour)

COURSES PRESENTED TO: 311-92B10 Medical Laboratory Procedures, Basic

PLACE: Classroom

REFERENCES: TM 8-227-5, Chapter 1,17 and attachment 3
Lennette, A.L., and others (ed) Manual of Clinical Microbiology. Bethesda; American Society for Microbiology, 1974

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: M 08-280-401-1, -2, and -3, issued at beginning of class.

PERSONNEL: One instructor, MOS 92B3H

INSTRUCTIONAL AIDS: Chalk, magnetic chalk board, eraser.

TROOP REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

I. INTRODUCTION (5 min)

A. Opening Statement: Welcome to Bacteriology. The study of microscopic organisms will be very interesting and enjoyable. The course is organized in building block fashion. Your responsibility in learning the material presented will be easier if you know how the course is organized, the manner in which the work is performed and what is expected of you.

B. Objective: Given a list of descriptions of bacterial cell structures, identify the structures and/or select, from a given list, the functions that they serve.

C. Class Procedure and Lesson Tie-in: First of 100 hours of instruction in Bacteriology

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*This lesson plan supersedes LP 08-280-401, 046

III. EXPLANATION (35 min)

- A. Orientation
  - 1. Welcome
  - 2. Introduction of staff
  - 3. Administrative policies
    - a. Duty hours
    - b. Breaks
      - (1) Lecture
      - (2) Practical exercise
    - c. Conduct
    - d. Appointments
    - e. Tardiness (Telephone number 221-5886)
    - f. Absences
  - 4. Details - see M 08-280-417-1
  - 5. Seating - as directed
  - 6. Study
- B. Overview of Course
  - 1. Manipulative skills
    - a. Specimen collecting
    - b. Culturing
      - (1) Streaking
      - (2) Subculturing
    - c. Decontamination
    - d. Staining

2. Decision skills
    - a. Selection of media and biochemical tests
    - b. Interpretation of tests and identification

C. Course Format

1. Sterilization and Decontamination
2. Media preparation
3. Developing techniques P.E. (Streaking and Morphology)
4. Gram stain
5. Cocci
    - a. Lectures and P.E.
    - b. Throat specimen
6. Enterics
    - a. Lectures and P.E.
    - b. Stool specimen
7. CSF specimen
8. Mixed specimen P.E.
9. Final mixed P.E.

NOTE: All practical exercises lead directly into the next in building block fashion.

D. Course Objectives

1. Crucial
    - a. S & D written
    - b. Cocci written
    - c. Enteric written
    - d. Gram stain (Interpretation and Performance)

e. Final mixed P.E.

2. Ranking - Final comprehensive written examination

E. Basic Terms

1. Bacteriology: The science devoted to the study of bacteria.

NOTE: Refer students to M 08-280-401-2 and -3 as definitions are given.

2. Bacterium: A living single-celled, microscopic organism which reproduces by binary fission and is classified in the Procaryotae Kingdom.

3. Petri dish: A plastic or glass dish which has a similar, but slightly larger dish for a top. Contains a semisolid layer of nutrients capable of sustaining bacterial growth without contamination. The nutrients are called media (singular: medium).

4. Colony: A collection of many bacteria whose visible macroscopic appearance is due to the reproduction of one bacterium.

5. Binomial nomenclature: A scientific naming procedure used in identifying microorganisms. It consists of two parts; the first is the genus, with the first letter capitalized and the entire word underlined. The second part is the species, which is also underlined but the first letter is not capitalized. For Example: Staphylococcus aureus.

6. Pathogen: An organism capable of producing disease.

7. Normal flora: Bacteria normally found in a certain part of the body and not harmful. They may cause disease if found in another part of the body; for example, the normal flora of the intestinal tract often cause disease of the urinary tract.

F. Bacterial Cell Structure

1. Capsule

a. Slimy, viscous coating

b. Associated with virulence

2. Cell wall

a. Shape

- b. Rigidity

3. Cytoplasmic membrane
    - a. Semipermeable
    - b. Allows passage of nutrients and waste products
4. Protoplasm - contents of the cell minus nuclear material
5. Cytoplasm - that portion of the protoplasm within the cytoplasmic membrane
6. Nuclear material
7. Spore
    - a. Dense, dehydrated
    - b. Resists environmental forces
8. Flagella

QUESTION: Which portion of the cell contributes to the virulence of the cell?

ANSWER: The capsule.

III. SUMMARY (5 min)

A. Review of Main Points

1. Orientation
2. Course format
3. Course objectives
4. Bacterial cell structure
5. Basic terms

B. Closing Statement: Clinical bacteriology is a diverse field. The methods, techniques and procedures taught in this course, if mastered, will produce adequate guidance for the performance of diagnostic microbiology.

SSC Richard A. Bean

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-401-1
LABORATORY SCIENCES DIVISION 046
MICROBIOLOGY BRANCH

Rules of the Bacteriology Laboratory

1. This is a bacteriology laboratory. Anything or everything in this room may be contaminated with pathogenic bacteria. Accordingly:

    a. Always wash your hands with an antiseptic soap after handling cultures of bacteria. Be sure to do so before going on "breaks."

    b. Wash the tops of the desks with Airkem when you begin and finish the day's practical work.

    c. Never put your fingers or foreign objects in your mouth, ears, nose, or eyes. Do not put items as pencils in your mouth.

    d. Do not bring food or drink into the laboratory/classroom.

    e. Smoking is not allowed in the bacteriology laboratory.

2. Avoid creating unnecessary noise during practical exercises.

3. This Branch is working on a tight schedule. You are to be seated in your assigned seat at the time classes are scheduled to start.

4. Anyone who has to leave the class for legitimate reasons must clear with the class leader and the chief instructor, sign out in the Microbiology office (Room 0308), and the administrative office (Room 1335).

5. The class leader is responsible for the discipline of the class. As students, you are responsible to the class leader. Assignments of details will be made by the class lead[illegible]

6. The area outside the classroom must remain clear of trash and will be policed after each break period, under the supervision of the class leader.

7. Reagents and antiseptics are kept in plastic squeeze bottles to facilitate their use. Anyone using these materials for other than their intended purpose will be referred to the Company Commander for disciplinary action.

8. You are studying to be medical laboratory specialists. Personal cleanliness and good grooming are mandatory. It is expected that enlisted men will appear cleanly shaven and with neat haircuts. All other aspects of good grooming (clean uniforms, shined shoes, clean brass, etc.) are required. Slovenly appearing individuals will not be tolerated.

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*This mimeo supersedes M 08-280-401-1, 035.

9. The materials in this laboratory are not to be removed from the room without permission of the instructor.

10. If a laboratory accident occurs, either with a specimen or yourself, notify the instructor immediately.

11. INDIVIDUAL COOPERATION IS IMPORTANT IF ALL OF YOU ARE TO BENEFIT FROM THE WORK DONE IN THIS COURSE. ALWAYS LISTEN CAREFULLY WHEN DIRECTIONS ARE GIVEN BY THE INSTRUCTOR AND FOLLOW THROUGH UNTIL THE TASKS ARE COMPLETED.

SFC Lyle J. Sorensen

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY M 08-280-401-2
LABORATORY SCIENCES DIVISION 026
MICROBIOLOGY BRANCH

Bacteriology Glossary

1. Acid-fast. Stained organisms resisting decolorization by acid-alcohol. Example: The Mycobacterium species are a group of acid-fast bacteria.

2. Aerobe. Organism which grows in the presence of free, atmospheric oxygen.

3. Anaerobe. Organism which grows in the absence of free, atmospheric oxygen; all oxygen requirements being satisfied by metabolism.

4. Antibiotic. Metabolic product of living microorganisms which are bacteriostatic or bactericidal against other microorganisms.

5. Antibody. Substance produced in the body in response to the introduction of an antigen; has the capacity to neutralize or to react with that specific antigen.

6. Antigen. Substance, usually protein or carbohydrate, which causes the production of an antibody when introduced into a suitable host.

7. Antiseptic. Bactericidal chemical agent which may be used topically on living tissue.

8. Aseptic Technique. Manipulative skills that prevent self-infections and/or contamination of laboratory specimens.

9. Bacterium. A living, single-celled microscopic organism which reproduces by binary fission.

10. Bactericidal. Irreversible action which results in the death of bacteria.

11. Bacteriostatic. Reversible action which inhibits the reproduction of bacteria.

12. Binomial Nomenclature. Scientific naming procedure used in identifying micro-organisms. It consists of two parts. The first part is the genus name with the first letter capitalized and the word underlined. The second part is the species name, which is also underlined; but the first letter is not capitalized. Example: Staphylococcus aureus.

13. Chemotherapeutic Agent. A chemical agent which kills infecting organism by "selective toxicity" is harmful to the infecting organism but relatively harmless to the host cells.

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*This mimeo supersedes M 08-280-401-2, 045

14. Colony. A collection of many bacteria whose visible macroscopic appearance is due to the reproduction of one bacterium.

15. Digestant. Mucolytic or proteolytic substance which is used to liquify a specimen. Example: A mucolytic agent is used on sputum specimens for tuberculosis studies.

16. Disinfectant. Bactericidal agent used on inanimate objects.

17. Endotoxin. Toxin which is liberated upon disintegration of a cell.

18. Exotoxin. Toxin excreted by a living cell.

19. Extracellular. Outside the cell.

20. Facultative. Organism which can adapt itself to growing in any oxygen environment.

21. Indicator. Any substance added to culture media to provide visual evidence of specific metabolic activity.

22. Inhibitor. Any substance which retards or stops the growth and reproduction of organisms.

23. Inoculate. To introduce microorganisms in or on a culture medium.

24. Intracellular. Within the cell.

25. Metabolism. The sum of all chemical reactions within the cell. All metabolism is controlled by enzymatic activity.

26. Microaerophile. Organism which grows in a reduced amount of free oxygen.

27. Motility. Exhibition of directional movement by a microorganism.

28. Normal Flora. Bacteria found in a certain part of the body and not harmful in that part of the body. They may be pathogenic if found in another part of the body.

29. Obligate. Limited to a specific environmental condition. Example: Obligate anaerobe.

30. Pathogen. An organism capable of causing disease.

31. Petri Dish. A plastic or glass dish which has a dish sitting on top. It contains a semi-solid layer of nutrients capable of sustaining

bacterial growth, without contamination. The nutrients are called Media.

32. Spore. Dehydrated body that may be formed by bacteria. This structure is highly resistant to adverse environmental forces.

33. Stain. A dye or reagent used to color organisms for purposes of microscopic observation.

34. Sterilize. To render free of all viable microorganisms and spores produced by certain microorganisms.

35. Thermolabile. Easily-altered, decomposed, or destroyed by heat.

36. Thermostable. Not easily altered, decomposed, or destroyed by heat.

37. Toxin. Metabolic by-product of certain organisms. Many toxins are capable of causing pathological changes.

38. Sterile. Free of all viable microorganisms and their spores.

SFC James A. Goode

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-401-3
LABORATORY SCIENCE DIVISION 038
MICROBIOLOGY BRANCH

Bacterial Cell Structure

1. The bacterial cell may be composed of the following parts:

a. Capsule. A slimy, viscous coating attached to and surrounding the cell wall of many bacteria. It is associated with virulence in that it interferes with phagocytosis.

b. Cell wall. Beneath the capsule, but outside the cytoplasm and cytoplasmic membrane, is the cell wall, a rigid structure giving the cell its characteristic shape. It is a complex substance consisting of amino acids, lipids, and polysaccharides.

c. Cytoplasmic (protoplasmic) membrane. Immediately beneath the cell wall lies a selective, semipermeable barrier that controls the passage of nutrients and waste products into and out of the cell. Location of cytochrome enzymes.

d. Cytoplasm. That portion of the bacterial protoplasm within the cytoplasmic membrane. Metabolic activity takes place in the cytoplasm.

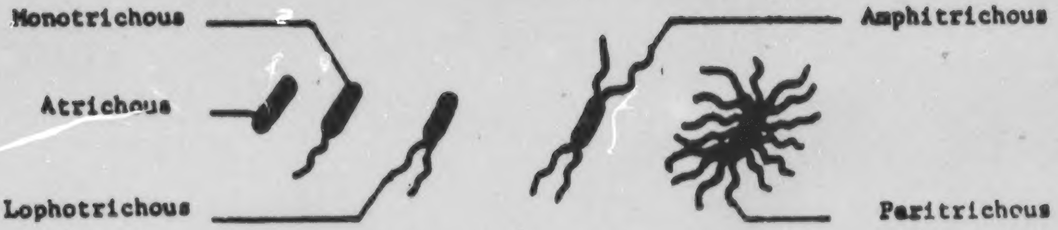
e. Protoplasm. The contents of the cell (cytoplasm) minus the nucleus.

f. Nucleus. Although bacteria have no distinct nucleus, certain "bodies" or particulate substances within the bacterial cell have a density and composition unlike that of the rest of the cytoplasmic mass. These structures have been interpreted as nuclei or nuclear material. This nuclear material consists of a particular type of substance--namely, deoxyribonucleic acid, which can be identified within the cells by specific nuclear staining reagents.

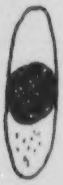
g. Flagella. Whiplike organs that render a bacterial cell motile. Organisms may be designated by the absence or the presence and position of these structures.

(1) Atrichous--having no flagella
(2) Monotrichous--having a single flagellum
(3) Lophotrichous--having two or more flagella in a tuft on one end
(4) Amphitrichous--having flagella on both ends
(5) Peritrichous--having flagella all around the bacterial cell

*This mimeo supersedes M 08-280-408, 046


Monotrichous
Atrichous
Lophotrichous
Amphitrichous
Peritrichous

h. Spore. A dense, dehydrated, highly refractile body formed by only two genera of bacteria (Bacillus and Clostridium) which is highly resistant to environmental forces and to most conventional staining methods. Spores are for the purpose of survival, and may be described as (A) central, (B) terminal, or (C) subterminal according to their location within a bacterial cell.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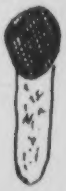
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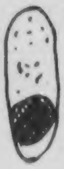
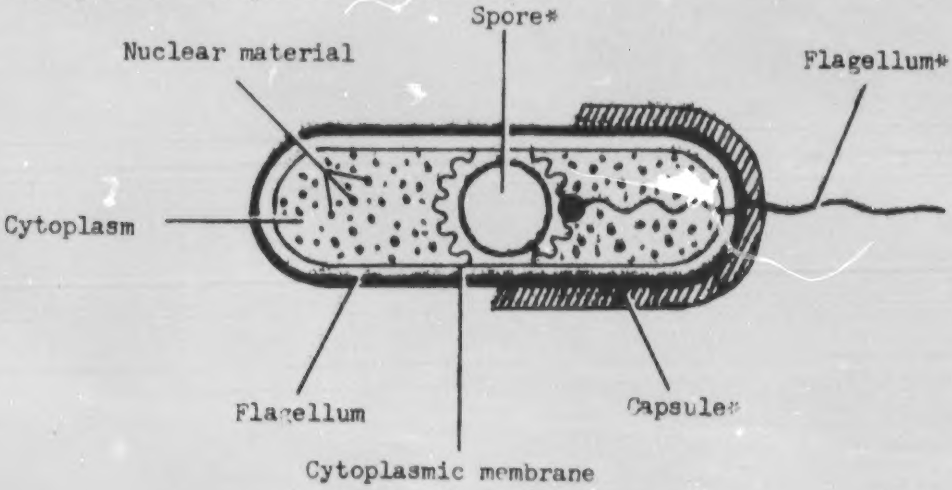

C

The above is a diagrammatic representation showing various locations of of spores in bacterial cells. In some cases the spore may be larger than the cell, causing the cell to have a bulb-like appearance. This is demonstrated by figure B above.

2. Example of a Composite Bacterial Cell


Spore*
Nuclear material
Flagellum*
Cytoplasm
Flagellum
Capsule*
Cytoplasmic membrane

SSG Gerald B. Howe

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-402
LABORATORY SCIENCE DIVISION 038
MICROBIOLOGY BRANCH

Sterilization and Decontamination - 2 hours

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Procedures, Basic

PLACE: Classroom

REFERENCES:

TM 8-227-5 Chap 4, pp 4-7.
Lennette, E.H. and others (eds). Manual of Clinical Microbiology. 2nd ed. Washington: American Society for Microbiology, 1974

STUDY ASSIGNMENT: None.

STUDENT UNIFORM AND EQUIPMENT: Not applicable.

TOOLS, EQUIPMENT, AND MATERIALS: M 08-280-401-1, prior to class.

PERSONNEL: One instructor, MOS 92B3H.

INSTRUCTIONAL AIDS: Chalk, chalkboard, eraser (see Annex D); magnetic placards 1 thru 18 (see Annex C); videotape #472 (see Annex A); slide projector and screen; S 08-280-402-1 thru -9 (see Annex B).

TROOP REQUIREMENTS: None.

TRANSPORTATION REQUIREMENTS: None.

METHOD OF INSTRUCTION: Lecture 1st hour; lecture and demonstration 2nd hour.

I. INTRODUCTION (5 min)

A. Opening Statement: Your main task in the bacteriology section is the cultivation, isolation, and identification of pathogenic bacteria. To accomplish this you must have sterile equipment and materials to insure that the patient's specimen is not contaminated by environmental bacteria.

B. Objectives

1. Given common laboratory situations involving sterilization/decontamination of media, equipment, and work surfaces, select the most correct means of accomplishing each.

2. Given a list of various thermolabile and thermostable solids and liquids, select the most correct method of

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*This lesson plan supersedes LP 08-280-402, 036.

sterilization/decontamination for each.

3. Given a typical laboratory situation, select the correct sterilization/decontamination procedures for waste disposal, accident clean-up, and work area preparation.

4. Given a situation, select the correct use of the autoclave for routine sterilization/decontamination procedures.

C. Class Procedure and Lesson Tie-in: Information presented in this block of instruction will be used throughout the course and is essential to its successful completion. This material will be tested by a crucial examination.

II. EXPLANATION (80 min)

A. Definitions

NOTE: Refer students to M 08-280-401-2.

1. Spore: A dehydrated body that may be formed by bacteria. This structure is highly resistant to adverse environmental forces.

2. Sterile: Void of all viable microorganisms and their spores.

3. Sterilization: Elimination of all viable microorganisms and their spores.

4. Decontamination: Making an object or area safe for unprotected personnel by absorbing, making harmless, or destroying bacteria with which they have been in contact.

5. Disinfectant: A compound which kills bacteria. Used on inanimate objects only.

6. Antiseptic: A compound which kills or inhibits bacteria, and may be used topically with no adverse effect on living tissue.

7. Thermolabile: Easily destroyed or decomposed by heat.

8. Thermostable:. Not easily effected by heat.

B. Methods of Sterilization

1. Autoclave

a. Principle: steam under pressure

b. Autoclave settings

  (1) Time - 15 minutes

  (2) Temperature - 121°C.

  (3) Pressure - 15 lb/sq. in.

  (4) Time should be extended if autoclave is packed completely full.

  (5) Temperature increases with pressure.

15 lb/sq. in. yields 121°C; 20 lb/sq. in. yields 126°C.

c. Most common method used in laboratory

  (1) Sterilize equipment and media for use

  (2) Used to decontaminate waste

d. Exhaust settings

  (1) Slow exhaust

    (a) Used for liquids - prevents boil over

    (b) Used for media preparation

  (2) Fast exhaust

    (a) Non-liquids

    (b) Waste material to be discarded

e. Limitations - thermolabile substances would be destroyed

  (1) Complex carbohydrates and urea

  (2) Plastic petri dishes and other plastic items which would melt

  (3) Blood and blood componants

f. Quality control

  (1) Autoclave tape

    (a) Indicates that items have been subjected to heat.

(b) Does not prove sterility

(2) Spore strips

(a) Paper strips saturated with spores

(b) Destruction of spores is the best method of indicating sterility

(3) Time and temperature charts

(a) Permanent record of autoclave use

(b) Provides evidence that autoclave is being used properly

(c) Laboratory registry requires them to be kept on file

g. Autoclaving items

(1) Disposable items - petri dishes (contaminated)

(a) Place in autoclave bucket

(b) Autoclave, fast or slow exhaust

(c) Dispose of in trash

(2) Non-disposable glass tubes

(a) Place in proper size rack

(b) Autoclave, fast or slow exhaust

(c) Empty, wash, and dry

h. Preparation of items for autoclaving (before use)

(1) Glass flasks for later use

(a) Cover mouth loosely with foil

(b) Label with autoclave tape - indicate date

(2) Gauze, rubber tubing, membrane filter apparatus

(a) Wrap in foil or paper

(b) Seal and label with autoclave tape - name of item and date

i. Safety

(1) Check pressure gauge before opening door

(2) Open door slowly to avoid steam

(3) Wear asbestos gloves to remove items

2. Hot air oven - dry heat

a. 170 to 180°C for 1½ to 2 hours

b. Used for thermostable solids

(1) Glassware, metal caps, glass test tubes, etc.

(2) Not for liquids - they would boil away

QUESTION: In what ways may thermostable solids be sterilized?

ANSWER: Autoclave (fast or slow exhaust) and hot air oven.

3. Filtration

a. Mechanical removal of bacteria and spores

b. Filter pore size ultra small (0.45 microns)

(1) Bacteria trapped on filter

(2) Liquids pass through into sterile container

c. Used for thermolabile liquids

(1) Complex carbohydrates and urea

(2) Serum

4. Gas sterilization

a. Ethylene oxide

b. Used for thermolabile solids

(1) Plastic materials such as petri dishes

(2) Surgical materials

5. Radiation - ultra violet light

a. Requires lenghty exposure time

b. Does not penetrate

c. Used in isolation hoods to help maintain sterility

NOTE: Avoid direct exposure of eyes and skin to ultra violet radiation. May cause severe burns.

6. Incineration (Bunsen burner flame)

a. Inoculating loops and needles

(1) Hold at 30 to 45° angle

(2) Flame from base of wire towards tip

NOTE: This avoids splattering of "boiling" bacteria

b. Forceps (for use with sensitivity discs)

(1) Dip in alcohol

(2) Ignite in burner and allow to burn freely

C. Methods of Decontamination

1. Disinfectants

a. Examples

(1) Phenol derivatives

(2) Quaternary ammonium compounds: A-33

NOTE: Stress that these compounds cannot be used on living tissue (see definition)

b. Uses

(1) Work area preparation

(a) Spray with disinfectant

(b) Wipe with paper towels

(2) Decontamination of pipets

(a) Soak in disinfectant with tip down

(b) Wash, dry and autoclave to re-sterilize

NOTE: Mention that pipets must be wrapped or placed in a canister to be autoclaved.

(3) Accident decontamination

(a) Cover/blot with paper towels

(b) Spray with disinfectant

(c) Wipe up spillage or breakage carefully

(d) Dispose of in autoclave bucket

(e) Report accident to instructor

c. Limitations

(1) Cannot be used topically on skin

(2) Cannot be added to media or reagents for use with live organisms

2. Antiseptics

a. Examples

(1) Soaps; pHisohex®

(2) Detergents

(3) Alcohol

b. Uses

(1) Wash hands after P.E. work

(2) Prevents self infection

(3) Preparation of venipuncture site

NOTE: Do not use on eyes, mouth, or mucous membranes

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038

D. Prevention of Self Infection

1. General

a. Do not put items in eyes, ears, nose, mouth

b. Avoid making aerosols out of liquid specimens

2. Eye contamination

a. Flush with water

b. Seek medical attention

NOTE: Do not use antiseptics or disinfectants in eyes. If these substances are accidently introduced, proceed as above.

QUESTION: What is used to prepare a venipuncture site; an antiseptic or a disinfectant?

ANSWER: An antiseptic is used, since disinfectants should not be used on skin (topically).

E. Demonstration - Videotape No. 472

1. Aseptic technique - Manipulatory skills that prevent self infections and contamination of laboratory or specimen.

2. Points to be observed

a. Preparation of work area

b. Labeling of media

c. Manipulation of materials

(1) Bacterial growth

(2) Plates

(3) Tubes

(4) Racks

(5) Loops and needles

(6) Pipets

(7) Forceps and susceptibility discs

3. Show VT No. 472 (11.36 minutes)

F. Questions From Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Definitions
2. Methods of sterilization/decontamination
3. Preparation for sterilization/decontamination in autoclave
4. Disposal of contaminated items
5. Work area preparation
6. Accident decontamination
7. Demonstration

B. Closing Statement: A crucial phase of microbiology is the knowledge of methods for killing, inhibiting, and removing microorganisms to insure that organisms identified were present only in the submitted specimens. By knowing and applying the methods learned today you can render a more effective service to the patient.

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ANNEX A
INSTRUCTIONAL AIDS

Videotape

TV No. 472, 11:36 minutes. Basic Aseptic Techniques.

Preparation of the work area and labeling of media to be inoculated are explained. Aseptic manipulation of bacteria, media, and various paraphenalia is stressed. The principles behind aseptic technique and its advantages are discussed. Dangers of not following principles of aseptic technique are also touched upon.

NOTE: This videotape is shown in Part II D-3.

ANNEX B
INSTRUCTIONAL AIDS

Slides (35mm)

S 08-280-402-1	Indicator tape before and after autoclaving
S 08-280-402-2	Membrane filtering apparatus
S 08-280-402-3	Incineration of a loop with a flame
S 08-280-402-4	A bottle of A-33
S 08-280-402-5	A disposable culture swab
S 08-280-402-6	An Erlenmyer flask covered with aluminum foil
S 08-280-402-7	13 x 100 mm test tubes in a 90 hole rack and culture tubes in a 40 hole rack
S 08-280-402-8	Wrapping a syringe for sterilization
S 08-280-402-9	Close up of front of autoclave

NOTE: These slides are shown where appropriate for explanation in part II.

ANNEX C
INSTRUCTIONAL AIDS

Magnetic Placards

MP 08-280-402-1	STERILIZATION AND DECONTAMINATION
-2	DEFINITIONS
--3	THERMOLABILE
-4	THERMOSTABLE
-5	METHODS OF STERILIZATION
-6	AUTOCLAVE
-7	AUTOCLAVE SETTINGS
-8	EXHAUST SETTINGS
-9	LIMITATIONS
-10	QUALITY CONTROL
-11	HOT AIR OVEN
-12	FILTRATION
-13	RADIATION
-14	INCINERATION
-15	METHODS OF DISINFECTION
-16	DISINFECTANTS
-17	ACCIDENT DECONTAMINATION
-18	ANTISEPTICS

NOTE: Use these magnetic placards where appropriate for explanation in Part II.

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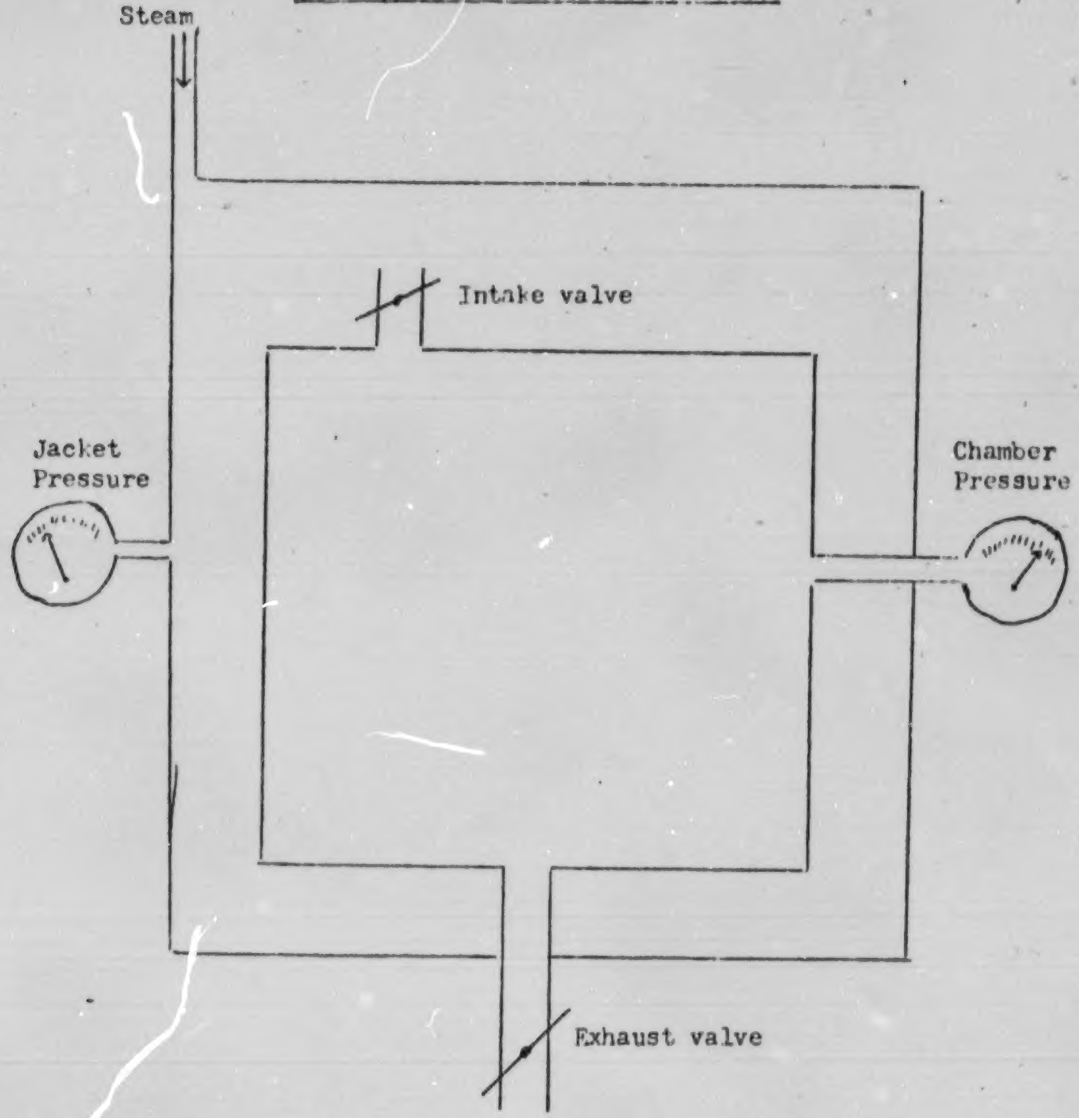
ANNEX D
INSTRUCTIONAL AIDS

Chalk Drawing Autoclave Schematic


Steam
Intake valve
Jacket Pressure
Chamber Pressure
Exhaust valve

NOTE: Use this chalk drawing to explain the function of the autoclave.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
MICROBIOLOGY BRANCH

M 08-280-402
038

Sterilization and Decontamination - 2 hours

The main task in the bacteriology section is the cultivation, isolation, and identification of pathogenic bacteria. To accomplish this you must have sterile equipment and materials to insure that the patient's specimen is not contaminated by **environmental bacteria.**

A very effective physical mechanism for sterilization is the autoclave, which employs the principle of steam under pressure. Water boils at 100° C under normal atmospheric pressure, and will get no hotter. If boiled under pressure, **however, the temperature will rise in proportion** to the amount of pressure. At 15 pounds per square inch the temperature reaches 121° C. Complete sterilization can usually be accomplished in 15 minutes at 15 pounds per square inch with the temperature at 121° C. Densely packed autoclave loads may require a few more minutes of autoclaving.

The autoclave is adaptable to a variety of laboratory uses, and is the most common method of sterilization used in the bacteriology laboratory. This instrument is used to sterilize media **and equipment prior** to their use, and to decontaminate media and equipment after being exposed to microorganisms. There are limitations to the use of the autoclave. It cannot be used to sterilize thermolabile substances, such as urea broth, complex carbohydrates, and cerain plastic materials.

The autoclave is equipped with two different exhaust settings; fast exhaust, and slow exhaust. Fast exhaust is used for non-liquids and waste materials. Slow exhaust is used for liquids, to prevent them from boiling over. A rapid decrease in pressure on a liquid which is hotter than its boiling point (as is the case in autoclave sterilization) causes the liquid to spontaneously boil over. For this reason, slow exhaust is the setting used for media preparation.

To make sure that an item has in fact been autoclaved, we have a quality control system which utilizes autoclave tape. This tape is heat sensitive and has diagonal lines which will turn dark when exposed to adequate heat and moisture. This change does not prove that an item is sterile, nor does it assure the technician of effective sterilization procedure. It only indicates that an item has been exposed to heat. To insure the efficiency of sterilization, the autoclave is checked at frequent intervals with spore strips. Spore strips are strips of filter paper impregnated with bacterial spores. A properly functioning autoclave will kill bacterial spores. Following autoclaving, the sporestrips are cultured for several days at various temperatures, negative results are indicative of a properly functioning autoclave. A third quality control measure is maintenance of time and temperature charts, which provide a a permanent record of autoclave use. Laboratory accreditation procedures require these charts be kept on file, since they provide evidence that the autoclave is being set and used properly. It should be noted, however, that these charts do not prove proper functioning of the autoclave. Of the three quality control measures, spore strips are the best single indication that the autoclave is functioning properly.

Specific items for autoclaving are treated in somewhat specific ways. Disposable items, such as contaminated Petri dishes and plastic culture tubes should be placed in stainless steel autoclave buckets and autoclaved on fast or slow exhaust prior to disposal in the trash. Non-disposable contaminated glass tubes should be placed in a proper sized rack and autoclaved on fast or slow exhaust. After cooling, they can be emptied, washed, dried, and returned to the shelf for reuse.

Items which must be sterilized prior to use are also treated in various ways, depending on the nature of the item. Glass flasks for later use should be labeled with autoclave tape with the date of autoclaving indicated. The mouth of each flask should be covered loosely with foil so that sterility will be maintained after autoclaving. Miscellaneous items, such as gauze, thermostable rubber tubing, and membrane filtering apparatus should be wrapped in foil or paper, and sealed with autoclave tape bearing the name of the item and the date autoclaved.

Since the autoclave uses "live" steam, several safety precautions must be observed. Check the pressure gauge before opening the door of the autoclave to insure that little or no pressure remains in the chamber. Open the door slowly to allow the remaining steam to escape. Asbestos gloves should be worn to remove items from the autoclave.

Another method of sterilization is the use of the hot air oven, which employs dry heat for sterilization. The hot air oven is used for thermostable solids, such as glass tubes or metal caps. A temperature of 170° C to 180° C for 1.5 to 2 hours is required to sterilize items using this method. Because of the high temperature and normal atmospheric pressure, this method is not acceptable for liquids, since they would boil away. It should be noted that non-liquid, thermostable items may be sterilized either by autoclaving, or by hot air sterilization, whichever is more convenient.

Filtration is a method for sterilization of liquids. Filtration mechanically removes bacteria and spores. This is accomplished by using a filter with an ultra-small pore diameter of approximately 0.45 microns. This pore size permits liquids to pass through the filter into a sterile container, while the bacteria and spores are trapped on the filter. Filtration is the method of sterilization used for thermolabile liquids such as urea broth, complex carbohydrates, and serum.

Certain gases such as ethylene oxide may be used as sterilizing agents for thermolabile solids. This method is used for sterilizing plastic Petri dishes before use, and is often used for surgical materials which cannot tolerate repeated exposure to high heat.

Radiation can also be used to sterilize materials. Ultraviolet light is often used in bacterial isolation hoods to help maintain sterility when the hood is not in use. Ultraviolet radiation cannot be used while the technician is working with the hood, since direct exposure of eyes and skin surfaces to ultraviolet radiation can cause severe burns. Other limitations of this method are the need for a lengthy exposure time, and the fact that ultraviolet radiation does not penetrate objects.

Direct heat may also be used as a method of sterilization/decontamination. In the laboratory we routinely use the Bunsen burner to sterilize inoculating loops and needles. The loop, or needle is held at a 30° to 45° angle to the flame. By flaming from the base of the wire toward the tip of the instrument we avoid splattering of bacteria. We also sterilize forceps by incineration, but the method is somewhat different. The tips of the forceps are dipped in alcohol, then ignited in the Bunsen burner flame and allowed to burn freely.

Many chemicals may be used for decontamination. They are usually referred to as disinfectants or antiseptics, depending on their toxicity to tissue and bacteria. An example of a disinfectant that is widely used in the laboratory is the quaternary ammonium compound A-33. Another common disinfectant is phenol and its derivatives. A detergent is an example of an antiseptic. Detergents may substantially remove bacteria by the use of an emulsifying agent. They may also contain additional compounds to increase their effectiveness, such as hexachlorophene in pHisohex, and iodine in Betadine. It should be noted that disinfectants cannot be used on living tissue. Disinfectants may be used to decontaminate work areas in preparation for bacteriological procedures. The area is sprayed with disinfectant, then wiped with paper towels. Another use of disinfectants is the decontamination of pipets. The pipets should be soaked tip down in the disinfectant. Later they may be washed, dried, and autoclaved for resterilization. Pipets should be wrapped or placed in a canister prior to autoclaving.

In the interest of safety a good general rule for bacteriology is to treat anything which spills as if it were contaminated. When spills occur, they should be covered with paper towels and sprayed with disinfectant. Allow the disinfectant to work for a short period of time, then carefully wipe up the material and dispose of it in an autoclave bucket. Students must report all accidents to an instructor.

As previously discussed, disinfectants cannot be used on the skin, and cannot be added to media or reagents. Antiseptics are less damaging to tissue, and may be used to decontaminate hands after working with bacteria. Antiseptics cannot be used in eyes, mouth, or mucous membranes. Antiseptics are also used to prepare venipuncture sites.

Since the technician is working with pure cultures of bacteria in the laboratory, it is necessary to use extreme care to avoid self-contamination. In general, items should not be put into eyes, ears, nose, or mouth. Aerosols are particularly dangerous to the technician and to those around the area. Care must be taken to avoid creating aerosols from liquid specimens. Eye contamination is especially dangerous, and should always be treated as an emergency. Flush the eye with large amounts of water and seek medical attention immediately should eye contamination occur. Do not use antiseptics or disinfectants in the eyes. If these substances are accidently introduced into the eyes, proceed as with bacterial contamination.

While working in the bacteriology laboratory one must maintain strict aseptic technique. Aseptic technique is a collection of manipulatory skills which prevent self-infection and contamination of the laboratory or the specimen.

A crucial phase of microbiology is the knowledge of methods for killing, inhibiting, and removing microorganisms to insure that organisms identified were present only in the submitted specimens. By knowing and applying these methods you can render a more effective service to the patient.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-403
LABORATORY SCIENCE DIVISION 038
MICROBIOLOGY BRANCH

Media Preparation (1 hour)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Procedures, Basic

PLACE: Classroom

REFERENCES:

GR 09-280-465-1
Difco Laboratories, Inc. Difco Manual of Dehydrated Culture Media and Reagents for Microbiological and Clinical Laboratory Procedures. 9th ed. Detroit: Difco Laboratories, Inc, 1971
Lennette, E.H. and others (ed). Manual of Clinical Microbiology. 2nd ed. Washington; American Society for Microbiology, 1974, chap 95.

STUDY ASSIGNMENT: None.

STUDENT UNIFORM AND EQUIPMENT: Not applicable.

TOOLS, EQUIPMENT, AND MATERIALS: Not applicable.

PERSONNEL: One primary instructor, MOS 92B3H.

INSTRUCTIONAL AIDS: Chalk, chalkboard, eraser, MP 08-280-403-1 thru -9 (see Annex A); VT No. 646 (see Annex B).

TROOP REQUIREMENTS: None.

TRANSPORTATION REQUIREMENTS: None.

TYPE OF INSTRUCTION: Lecture.

I. INTRODUCTION (5 min)

A. Opening Statement: In order to cultivate successfully and identify the medically important bacteria, the technician must have media on which he can rely. In many cases the technician must prepare this media himself. For this reason, one must be proficient in the basic techniques employed in media preparation.

B. Objectives

1. Given a situation, select the correct steps in preparing bacterial media and the correct steps required to quality control that media.

2. Given a list of various types of media, select the correct type of media based on agar consentration.

3. Perform the calculations required to prepare media as demonstrated in class.

C. Class Procedure and Lesson Tie-in: Methods for preparing various types of media will be discussed, followed by a practical exercise in media preparation. A videotape will demonstrate media preparation.

II. EXPLANATION (30 min)

A. Media - Provides nutrients used to support the growth of bacteria in the laboratory.

1. Uses

a. Cultivation of bacteria

b. Isolation of bacteria

c. Differentiation of bacteria

B. Physical Types

1. Agar

a. Solidifying agent

b. Physical properties

(1) Melting point - 99°C

(2) Solidifying point - 40°C

(3) Dispensing temperature - 45 to 50°C

2. Solid media - 1.5 to 2% agar

3. Semisolid media - 0.2 to 0.4% agar

4. Liquid (broth) media - usually no agar

NOTE: Agar concentration determines physical type of media.

C. Preparation of Media

1. Directions given on bottle
    a. Grams of dehydrated media required for 1 liter.
    b. Rehydration procedure
    c. Heating or boiling (if needed)
    d. Method of sterilization
    e. Other special instructions
2. Calculation of requirements
    a. Grams required for 1,000 mls given in directions
    b. Determine amount to be prepared
    c. Divide or multiply number of grams according to proper ratio
    d. Give example
3. Equipment and materials
    a. Dehydrated media
    b. Balance
    c. Spatula
    d. Weighing boats
    e. Graduated cylinder
    f. Erlenmeyer flask
        (1) Clean and dry
        (2) Twice the volume of media to be prepared
4. Weigh media carefully
5. Rehydrate media with distilled or deionized water
6. Boil into solution (if necessary)

NOTE: Media containing agar must be boiled into solution.

7. Sterilization
    a. Filtration - thermolabile ingredients
    b. Autoclave - Follow directions for time and temperature
8. Dispensing
    a. Plate media
        (1) After autoclaving
        (2) Cool to pouring temperature - 45 to 50°C
        (3) Add sterile thermolabile ingredients if needed
        (4) Pour into petri dishes
            (a) 60 plates per liter
            (b) 16 ml per plate
    b. Tubed media
        (1) Dispence correct amount into tubes and cap
        (2) Label
        (3) Autoclave at recommended time and temperature
        (4) Slant if necessary

D. Quality Control
1. Plates - incubate at 37°C for 18-24 hours
    a. Sterility check
    b. Dries surface of media
2. Tubes
    a. Batch testing
    b. Run known cultures

E. Storage - refrigerate at 4-6°C

F. Demonstration (Videotape)

  1. Points to observe

  2. Show VT 646 (22 min)

  3. Answer questions

G. Questions from Students

QUESTION: What are the routine time, temperature and pressure settings for autoclaving?

ANSWER: 15 minutes, 121 degrees Celcius, 15 psi.

QUESTION: Why are plates incubated?

ANSWER: Petri dishes are incubated to check for bacterial and fungal contaminants which may inadvertantly be in or on the media during or after preparation.

III. SUMMARY (5 min)

A. Review of Main Points

  1. Media

  2. Physical types of media

  3. Preparation of media

  4. Quality control

  5. Storage

  6. Demonstration

B. Closing Statement: As basic laboratory specialists, you will be called upon to prepare media for use in the clinical bacteriology laboratory. The correct and consistant preparation of this media is essential to the isolation and identification of bacteria from clinical specimens.

INSTRUCTIONAL HOURS

Number of instructors required - - - 1

Primary hours	1
Secondary hours	0
Total	I

ANNEX

ANNEX A
INSTRUCTIONAL AIDS

Magnetic Placards

MP 08-280-403-1	MEDIA PREPARATION
-2	PURPOSE
-3	PREPARATION
-4	CALCULATION OF REQUIREMENTS
-5	EQUIPMENT AND MATERIALS
-6	PREPARATION OF MEDIA
-7	PLATE MEDIA
-8	TUBE MEDIA
-9	QUALITY CONTROL

ANNEX B
INSTRUCTIONAL AIDS

Videotape

VT No. 646, 22 minutes. Media Preparation

The preparation of media is demonstrated. The various techniques for the preparation of special media are explained. The use of the different equipment in media preparation, as well as the more common errors, are discussed.

The videotape begins with a brief discussion of dehydrated media, selection of flasks, deionized water, balance, weighing boats, and spatula, test tubes and racks, magnetic stirrer, mechanical and hand dispensers, slant racks, burner, tripod, and asbestos gloves, Petri dishes, membrane filter apparatus, autoclave, indicator tape, water baths, and walk-in refrigerator and incubator. The preparation of tubed media is demonstrated. Weighing, dissolving, dispensing, and labeling is demonstrated in detail. The preparation of plating media is then shown, with emphasis on pouring the plates. The special procedures of preparing blood agar plates are demonstrated prior to a detailed demonstration of the use of the membrane filter apparatus to sterilize thermolabile liquids.

NOTE: This videotape is shown in part II, F.

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SSG Richard A. Bean

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-404
LABORATORY SCIENCES DIVISION 046
MICROBIOLOGY BRANCH

Media Preparation (6 hours)

COURSES PRESENTED TO: 311-92B20, Medical Laboratory Procedures (Basic)

PLACE: Laboratory

REFERENCES:

TM 8-227-5, chap 4.
Frobisher, M. Fundamentals of Microbiology. 8th ed. Philadelphia: W. B. Saunders Company, 1968, 4.1 to 4.2.
Difco Laboratories, Inc. Difco Manual of Dehydrated Culture Media and Reagents for Microbiological and Clinical Laboratory Procedures. 9th ed. Detroit: Difco Laboratories, Inc. 1971.

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: See Annexes B and C

PERSONNEL: Eight instructors (MOS 92B4H)

INSTRUCTIONAL AIDS: See Annexes A, B, and C

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Practical exercise; conference

I. INTRODUCTION (5 min)

A. Opening Statement: Media preparation requires the application of principles which were explained during the lectures covering media and reagents, sterilization, and aseptic technique. The methods involved in suitable media preparation must be thoroughly mastered for the proper isolation and identification of bacteria.

B. Objectives

Prepare bacteriological media suitable for student use.

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*This lesson plan supersedes LP 08-280-404, 065.

C. Class Procedure and Lesson Tie-in

1. Individuals will prepare specific types of media assigned by the instructors.

2. This 6-hour block, conducted as two 3-hour practical exercises, will be used to prepare students for the media preparation crucial objective.

II. APPLICATION (260 min)

A. Instructors' Activities

1. Specific duties assigned to students

NOTE: See Annex A.

2. Materials issued to students

NOTE: See Annex B and C.

3. Safety regulations explained

4. Classroom control maintained

5. Errors corrected

6. Questions answered

7. Supervise cleanup of all facilities

B. Students' Activities

1. Preparation of media as directed

a. Adherence to standard laboratory technique

b. Adherence to safety regulations

2. Performance of cleanup as directed

III. CRITIQUE (5 min)

A. Questions From Students

B. Review of Main Points

1. Students' general laboratory technique

2. Students' preparation of media

3. Students' discrepancies clarified

C. Closing Statement: The media you have prepared will be used during the course. In large measure, the quality of this media will be responsible for your success or failure in isolation and identificaiton of bacterial organisms. Correct now any of your errors made apparent by this exercise, so that your competence in later assignments to bacteriology laboratories will be enhanced.

ANNEX A
INSTRUCTIONAL AIDS

Requirements for Media Preparation

The successful completion of this block of instruction requires careful coordination and advance planning among all of the basic bacteriology instructors. All students must be involved in this exercise. Individual students should be assigned the preparation of 250 ml or half-liter volumes of plate media, and be required to participate in all steps from weighing the dehydrated product through dispensing to Petri dishes, labeling, and storage. Individual students will also be responsible for all phases of preparation of the various types of tube media. The instructors are responsible for locating and marking available, prior to class, all items needed to complete the exercise.

NOTE: This Annex is used in II.A.

ANNEX B
INSTRUCTIONAL AIDS

Equipment and Supplies

1.	Autoclave	1 per class
2.	Refrigerator	1 per class
3.	Incubator	1 per class
4.	Waterbath, electric, serological	2 per class
5.	Balance, weighing	6 per class
6.	Weighing boat, plastic	50 per class
7.	Spatula, media transfer	6 per class
8.	Flasks, Erlenmeyer, 1 and 2 liter sizes	as required by Annex C
9.	Test tubes, 13 x 100 and 16 x 125	as required by Annex C
10.	Closures, test tube	as required by Annex C
11.	Racks, test tube, 40 and 90 holes	as required by Annex C
12.	Petri dishes, plastic, sterile	as required by Annex C
13.	Media	as required by Annex C
14.	Sheep blood, sterile, defibrinated	as required by Annex C
15.	Slanting racks	as required by Annex C
16.	Broth dispensers	6 per class
17.	Metal foil, roll	1 per class
18.	Magnetic stirrer	1 per class
19.	Magnets	18 per class
20.	Tape, pressure sensitive, roll	1 per class
21.	Markers (wax and felt tip)	12 per class
22.	Burner, Fisher, or Meeker type	24 per class
23.	Tripod	24 per class
24.	Burner pad, asbestos	24 per class

NOTE: This Annex is used in II.A.

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ANNEX C
EQUIPMENT AND SUPPLIES

Media Requirements

MEDIA	QUANTITY* (50 students)		
	1st 3 hours	2d 3 hours	Media prep** crucial obj.
1. Mannitol	3.5 L	0	0
2. Eosin methylene blue agar	0	4.0 L	3.25 L
3. Salmonella-Shigella agar	0	4.0 L	3.0 L
4. MacConkey agar	0	4.0 L	10.25 L
5. Triple sugar iron agar	3.75 L	0	2.0 L
6. Glucose (semisolid)	0	1.5 L	0
7. Phenylalanine deaminase agar	0	0	2.5 L
8. Lysine decarboxylase broth	0	2.0	0
9. Malonate broth	0	2.5 L	0
10. Tryptose broth (Indole)	0	0	1.5 L
11. Citrate agar	0	0	1.5 L
12. Bile esculin azide slants	2.5 L	0	0
13. Practice agar (TWA)	1.75	0	0
14. Mueller-Hinton agar	1.0 L	0	0
15. Nutrient agar slants	0	0	5.0 L

NOTE: This annex is used in II.A.

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*See pg C-2 for requirements per class of 50 per practical exercise.

**This media is to be made in the fourth week of the course to satisfy the crucial objective in LP 08-280-435.

Media requirements per class of 50 per practical exercise.

		Practical exercise							
		407	416	419	421	429	431	433	436
1.	Sheep blood agar plates	350	350	0	100	225	188	350	350
2.	Mannitol salt agar plates	50	25	0	0	15	12	50	50
3.	Eosin Methylene blue agar plates	0	0	0	300	10	12	50	50
4.	Salmonella-Shigella agar plates	0	0	0	300	5	8	50	50
5.	MacConkey agar plates	0	0	0	300	75	72	300	200
6.	Triple sugar iron agar tubes	0	0	0	300	130	138	250	300
7.	Glucose agar (semisolid) tubes	0	0	0	100	5	8	100	100
8.	Phenylalanine deaminase agar tubes	0	0	0	225	5	4	200	200
9.	Lysine decarboxylase broth tubes	0	0	0	150	5	4	150	150
10.	Malonate broth tubes	0	0	0	200	5	4	200	200
11.	Indole test medium tubes	0	0	0	50	5	4	100	150
12.	Urea broth tubes	0	0	0	225	5	4	200	200
13.	Citrate agar tubes	0	0	0	75	5	4	150	150
14.	Bile esculin azide tubes	50	150	0	0	5	4	200	200
15.	Mueller-Hinton agar plates	0	0	50	0	0	0	0	0
16.	Coagulase test medium tubes	50	50	0	0	5	4	100	100
17.	Practice agar (TWA)	100	0	0	0	0	0	0	0

NOTE: This Annex is used in II.A.

NOTE: Media prep #1 is performed prior to PE 407, media pre #2 is performed prior to PE 419, and the crucial objective media preparation is performed prior to PE 436.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-405
LABORATORY SCIENCES DIVISION 038
MICROBIOLOGY BRANCH

Nutrition and Cultivation of Bacteria (1 hour)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Procedures, Basic

PLACE: Classroom

REFERENCES:

Bailey, W. R., and Scott, E. G., Diagnostic Microbiology. 4th ed., St. Louis, C. V. Mosby Co., 1974, Chap 1.

Lennette, Edwin, and Truant, Joseph. (Eds). Manual of Clinical Microbiology. 2d Ed. Bethesda, MD: American Society for Microbiology, 1974, Chap 6.

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: None

TOOLS, EQUIPMENT, AND MATERIALS: None

PERSONNEL: One primary instructor (MOS 92B3H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, and eraser; overhead projector; screen; T 08-280-405-1 thru -3 (see Annex A); MP 08-280-405-1 thru - 10 (see Annex B)

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

I. INTRODUCTION (5 min)

A. Opening Statement: Identification of bacteria is dependent upon successful growing or cultivation. Providing the required enviromental and nutritional factors will insure sufficient growth for identification.

B. Objectives

1. Given a list of nutritional and enviromental requirements of medically important bacteria, select the requirements that are needed for bacterial growth.

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*This lesson plan supersedes LP 08-280-405, 025

2. Given a growth curve, select the name of the various growth phases and select the phase best suited for biochemical evaluation of the growth in Clinical Bacteriology.

3. Given a source of nutrition, select the pH (acid or alkaline) resulting from the bacterial utilization of that source.

4. Given a list of oxygen requirements and bacterial growth patterns in those oxygen environments, select the correct oxygen requirement for those bacteria.

5. Given a list of media contents, select the correct classification of those media based on those contents.

C. Class Procedure and Lesson Tie-in

Nutritional and environmental requirements will be considered in all practical exercises to correctly process various clinical specimens. When these requirements are met, the growth of organisms found in simulated specimens will be enhanced.

II. Explanation (30 min)

A. Nutritional Requirements

1. Water
2. Carbohydrates (sugars)
3. Proteins (peptones)
4. Salts
5. Trace elements
6. Amino acids
7. Vitamins

NOTE: Not all bacteria require all listed nutritional requirements

B. Environmental Requirements

1. Oxygen

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a. Aerobic environment

(1) Free oxygen present

(2) Organisms recovered: Aerobes

(3) This environment is produced in walk-in incubator

b. Microaerophilic environment

(1) Reduced oxygen concentration

(2) Organisms recovered: Microaerophiles

(3) This environment is produced in CO_2 incubator

QUESTION: Which oxygen environment, microaerophilic or aerobic, has the lowest free oxygen concentration?

ANSWER: The microaerophilic oxygen environment.

c. Anaerobic environment

(1) No oxygen present

(2) Organisms recovered: Anaerobes

(3) This environment is produced in anaerobe jar

NOTE: Organisms that will grow in any oxygen environment are termed facultative.

2. Moisture: high relative humidity

3. Temperature: 35° - 37° C

4. pH: 6.8 - 7.4

C. Growth Curve

NOTE: Show T 08-280-405-1

1. Lag Phase: organisms adapt to new environment

2. Log Phase: exponential growth

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- a. Bacteria demonstrate diagnostic characteristics
- b. Phase most often worked with in Microbiology

3. Stationary Phase: viable population count is constant

4. Death Phase: viable population is decreasing

D. Sources of Nutrition and Resulting pH.

NOTE: Show T 08-280-405-2 and T 08-280-405-3.

1. Carbohydrate utilization yields acid pH

2. Protein utilization yields alkaline pH

E. Media Classification

1. Basal media
    - a. Contain minimal media to support the growth of non-fastidious organisms
    - b. Proteins are sole source of nutrients
    - c. Example: Trypticase Soy Medium

2. Enriched media
    - a. Contain basal media plus enrichments
    - b. Sheep blood most common enrichment
    - c. Allows growth of fastidious organisms
    - d. Example: Sheep Blood Agar

3. Differential media
    - a. Contain basal media, a differential metabolite, and an indicator
    - b. Differentiate bacteria on the basis of metabolite utilization
    - c. Example: Triple Sugar Iron Agar 953

4. Differential selective media

    a. Contain basal media, differential ingredients, and inhibitors

    b. Inhibit growth of certain bacteria

    c. Posses differential capabilities

    d. Example: Mac Conkey Agar

QUESTION: Which class of media would be best suited for the cultivation of fastidious organisms?

ANSWER: Enriched Media

III. SUMMARY (5 min)

A. Review of Main Points

1. Nutritional requirements

2. Environmental requirements

3. Growth curve

4. Sources of nutrition and resulting pH

5. Media classification

B. Closing Statement: Bacteria provided with required nutritional and environmental factors will grow on various media in-vitro. Once growth is established identification can be sought by various biochemical testing procedures.

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ANNEX A
INSTRUCTIONAL AIDS

Transparencies

T 08-280-405-2	Diagram demonstrating the effects of bacterial metabolism of a carbohydrate substrate.
T 08-280-405-3	Diagram demonstrating the effects of bacterial metabolism of a protein substrate.
T 08-280-405-1	Diagram of bacterial growth curve.

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ANNEX B
INSTRUCTIONAL AIDS

LP 08-280-405
038

Magnetic Placards

MP 08-280-405-1	NUTRITION AND CULTIVATION OF BACTERIA
MP 08-280-405-2	NUTRITION REQUIREMENTS
MP 08-280-405-3	ENVIRONMENTAL REQUIREMENTS
MP 08-280-405-4	GROWTH CURVE
MP 08-280-405-5	SOURCES OF NUTRITION AND RESULTING pH
MP 08-280-405-6	MEDIA CLASSIFICATION
MP 08-280-405-7	BASAL
MP 08-280-405-8	ENRICHED
MP 08-280-405-9	DIFFERENTIAL
MP 08-280-405-10	DIFFERENTIAL SELECTIVE

SSG Richard A. Bean

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-405-1
LABORATORY SCIENCES DIVISION 046
MICROBIOLOGY BRANCH

Oxygen Requirements of Microorganisms

Organisms are classified according to their oxygen requirements.

1. Aerobes--require normal atmospheric oxygen.
2. Microaerophiles--require reduced atmospheric oxygen.
3. Anaerobes--require atmosphere devoid of free oxygen.
4. Facultative anaerobes--adapt to any oxygen environment.

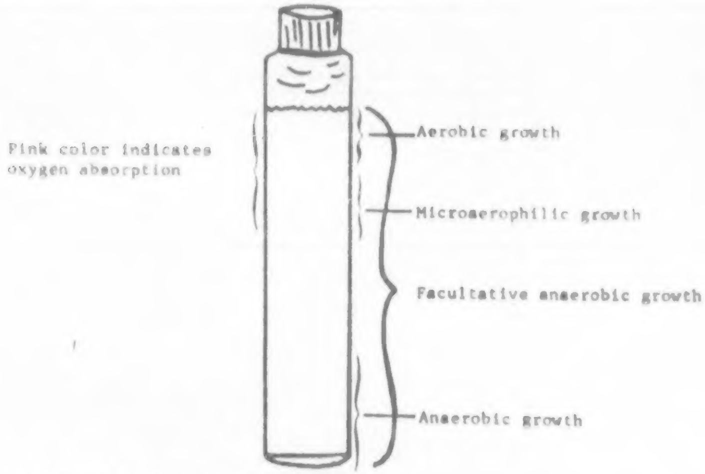
One may attach the adjectives "obligate" and "facultative" to the above terms to expand their meaning. Obligate means strict or absolute while facultative means flexible: therefore an obligate anaerobe is a strict or absolute anaerobe and can never be anything else.

Most pathogens are aerobes, but their growth is enhanced in the presence of an increased CO_2 environment. Care must be exercised not to confuse the terms "aerogenic" and "anaerogenic" with "aerobic" and "anaerobic."

THIOGLYCOLLATE BROTH

Thioglycollate broth is a very useful tool to the bacteriologist since this broth medium supplies the spectrum of oxygen requirements from aerobic to anaerobic in the same tube. This unusual feat is accomplished by the chemistry of the medium with the inhibition of the absorption of atmospheric oxygen.

Resazurin is placed into the medium as an indicator for oxygen. This indicator turns the medium pink-red when unwanted oxygen is absorbed into the thioglycollate. It the tube we are using contains this pink color in the upper 1/3 of the broth, it must be re-autoclaved or boiled before use, or discarded.


Pink color indicates oxygen absorption
Aerobic growth
Microaerophilic growth
Facultative anaerobic growth
Anaerobic growth

*This mimeo supercedes M 08-280-405-1, 035

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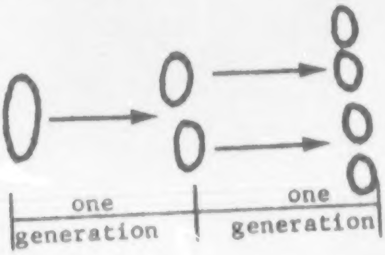
M 08-280-405-1
046

Growth and Reproduction of Bacteria

GENERATION TIME--the time required for one cell to produce two daughter cells.

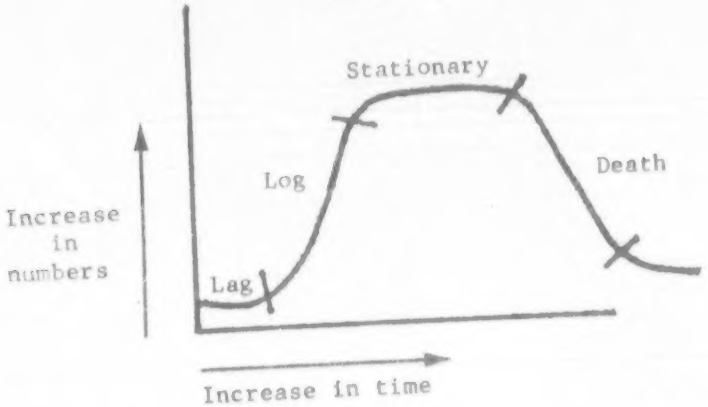


The generation time is characteristic of the species. For example:

Escherichia coli----15 minutes
Tubercle bacillus---20 hours
Treponema pallidum--30 hours

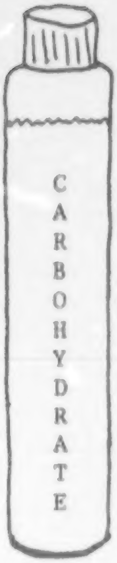
As bacteria grow and reproduce we find that a particular pattern of growth results which can be illustrated by a graph. Each portion of the resulting curve is significant and consequently each phase has a special terminology.

GROWTH CURVE



The optimum time for working with a particular bacterial culture is during the Log phase of growth, or that portion between 18 to 24 hours. Every organism has a similar growth curve depending upon its generation time.

Products of Enzymatic Reactions

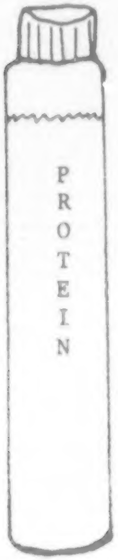


→ AEROBIC → ACID pH
CO_2
H_2O
ENERGY
UNSTABLE ACID

→ ANAEROBIC → ACID pH
CO_2
H_2
STABLE ACIDS

NO UTILIZATION

NEUTRAL pH



→ AEROBIC → ALKALINE pH
NH_3
CO_2
H_2O

→ ANAEROBIC → ALKALINE pH
AROMATIC AMINES

NO UTILIZATION

NEUTRAL pH

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-406
LABORATORY SCIENCES DIVISION 038
MICROBIOLOGY BRANCH

Inoculation, Streaking of Media, and Colony Morphology (2 hours)

COURSES PRESENTED TO: 311-92B20, Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES:

Bailey, W. R. and Scott, E. G. Diagnostic Microbiology. 4th ed. St Louis: C. V. Mosby Co., 1974. Chap 4.
Lennette, E. H. and others (ed). Manual of Clinical Microbiology. 2d ed. Washington: American Society for Microbiology, 1974. Chap 6.

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: GR 09-280-465-1 Prior to class

PERSONNEL: One instructor, MOS 92B3H

INSTRUCTIONAL AIDS: Chalk, magnetic chalkboard, and eraser; videotape TV #188, TV recievers, (see Annex A); magnetic placards (see Annex B)

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

I. INTRODUCTION (5 min)

A. Opening Statement: The ability to grow pathogenic bacteria on artificial media is of little value in the clinical microbiology laboratory unless a method for separating the various species in the bacterial culture is available. The use of proper inoculating and streaking procedures will enable you to accomplish this segregation. The procedure used in the clinical laboratory to separate mixed cultures is called "Streaking for Isolation." We use the "four quadrant" method to achieve this isolation.

B. Objectives

1. Given a clinical bacteriology situation, select the correct method of inoculating tube and plate media using the loop, needle, and swab.

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*This lesson plan supersedes LP 08-280-406, 046

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2. Given a list select the correct steps in the performance of the "four quadrant" streaking method.

3. Given a clinical bacteriology situation, select the steps used for subculture or re-isolation of a bacterial growth.

4. Given a description of a bacterial colony growing on any of the plated media taught in class, select the correct colony morphology for those bacteria.

C. Class Procedure and Lesson Tie-in

1. The first hour of this lecture is given before inoculating media for PF 08-280-407. The second hour is presented prior to the time the students read the colony morphology of the plates they inoculated earlier.

2. Practice in performing inoculating, streaking, selecting isolates, subculturing, and re-streaking follows this period of instruction. Your ability to carry out these procedures will be graded later in this course.

II. EXPLANATION (35 min)

A. Inoculation of Media

NOTE: Stress aseptic techniques.

1. Inoculation loop

a. Purpose

(1) Inoculation of fluid specimens to media

(2) Transfer small amounts of liquids

(3) Streaking inoculated plate media

b. Procedure

(1) Inoculation: obtain a small anount of fluid and spread over area of the first quadrant

(2) Streaking: (to be coverd later in the lecture)

2. Inoculation needle

   a. Purpose

      (1) Used for transferring colonies

      (2) Used for inoculating tubed media

   b. Procedure for inoculating plate media

      (1) Touch only the top center of the colony to be transferred

      (2) Make three "s" streaks near the edge of the plate in the area of the first quadrant.

      (3) Rotate the needle between the "s" streaks

      (4) Streak with a loop

   c. Procedure for inoculating tubed media

      (1) Liquid medium

         (a) Touch the tip of the needle containing the inoculum to side of tilted tube

         (b) Assure inoculum is below surfacr of medium when tube is turned upright

         (c) Gently mix tube of medium by agitation

      (2) Solid medium (slant)

         (a) Stab the butt to botton of the tube

         (b) Streak the slant with "fishtail" pattern

      (3) Semisolid medium

         (a) Stab into the medium one half the depth of the medium in the tube

         (b) Be sure to stab straight in and straight out

3. Sterile swab

   a. Purpose

(1) Direct collection of clinical specimens

(2) Direct inoculation of specimen to media

b. Procedure

(1) Roll swab over surface of first quadrant area

(2) Insure inoculum is representative of the source

NOTE: If the specimen is submitted on a dual swab inoculate one side to the first quadrant area, then inoculate the other side by making three "s" streaks with the swab.

B. Streaking

1. Four quadrant method

a. Sterilize instruments

QUESTION: What method is used for sterilization of loops and needles?

ANSWER: Incineration.

b. Allow loop to cool

c. Streak plate

NOTE: Refer to TR 08-280-465-1

(1) Proximity of lines

(2) Re-enter the previous quadrant 4 times

(3) Use maximum surface area

(4) Once inoculum is obtained do not re-enter

(5) Do not cut medium

2. Progressive dilution of bacteria obtained by streaking

3. Isolated colonies obtained because of individual bacterial cells

C. Videotape (see annex B)

1. Points to be observed

a. Selection of instruments

b. Inoculation methods

c. Sterilization of instruments

d. "Four quadrant method"

e. Common errors

f. Re-streaking

g. Subculturing

2. Show videotape

SECOND HOUR (40 min)

D. Colony Morphology

1. Sheep blood agar (SBA)

a. Hemolysis

NOTE: Hemolysis is the lysis or RBC's caused by the hemoysins secreted into the medium by organisms

(1) Alpha-green zone

(a) RBC's intact

(b) Medium beneath colony and around it turns green

(2) Beta-clear zone

(a) RBC's destroyed

(b) Medium reverts to original color of base (straw or yellow)

(c) Entire plate may change

(3) Gamma-no zone

- b. Size
  - (1) Small
  - (2) Medium
  - (3) Large
- c. Color
  - (1) White
  - (2) Gray
  - (3) Yellow

2. Differential media
   - a. Color change due to:
     - (1) Metabolite utilization
     - (2) pH change
     - (3) pH indicator

NOTE: Differential media will be taught at a later date

3. Importance of colony morphology
   - a. Identify different types of colonies
   - b. Determine success of isolation
   - c. Differentiation for testing purposes

E. Subculturing

1. Used when few colonies are available
   - a. Provides pure culture for testing purposes
   - b. Part of some test procedures
2. Contamination
   - a. Most common error
   - b. Failure to obtaine pure culture

3. Procedure

   a. Selection of colony

      (1) Identify by colony morphology

      (2) Must be isolated colony

      (3) Use one colony only

   b. Transfer colony

      (1) Use needle

      (2) Touch top center of colony

   c. Inoculate medium

      (1) Use needle

      (2) Make three "s" streaks

   d. Streak plate with loop

   e. Incubate for 18 to 24 hours at 37 C

   f. Observe subculture

      (1) Compare to original colony

      (2) Check for contamination

NOTE: If a pure culture is not obtained on a subculture care must be taken that the correct colony type is used for any testing to be performed.

F. Re-streaking-used when isolation is not obtained on the original plate medium.

III. SUMMARY (5 min)

A. Review of Main Points

1. Inoculation of media

2. Streaking

3. Colony morphology

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4. Subculture

5. Re-streaking

B. Closing Statement: All the techniques which were discussed and demonstrated must be mastered if bacteria are to be successfully isolated from clinical specimens and subsequently identified.

ANNEX A
INSTRUCTIONAL AIDS

Videotape

TV #188, 15:08 minutes. Streaking for Isolation

The uses of sterile swabs and the bacteriological inoculating loop and needle are explained. Various methods for inoculating plate media are shown, followed by a detailed discussion and demonstration of the use of the loop for streaking plate media for isolation using the "four quadrant" method. The results of poor streaking and correct streaking are shown using plates with fully developed colonies. The correct method for selecting and transferring individual isolated colonies by means of the inoculating needle is explained and demonstrated followed by a discussion and demonstration of re-isolation when isolated colonies are not present.

NOTE: This videotape is shown in part IIC2.

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ANNEX B
INSTRUCTIONAL AIDS

Magnetic Placards

1. INOCULATING MEDIA
2. STREAKING
3. RESTREAKING
4. SUBCULTURING
5. COLONY MORPHOLOGY

NOTE: Use these magnetic placards where appropriate to explanation.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *IM 08-280-406
LABORATORY SCIENCES DIVISION 038
MICROBIOLOGY BRANCH

Streaking, Selection, and Morphology of Isolates

The ability to grow pathogenic bacteria on artificial media is of little value in the clinical microbiology laboratory unless a method for separating the various species of the bacterial culture is available. The use of proper inoculating and streaking procedures will enable you to accomplish this segregation. The procedure used in the clinical laboratory to separate mixed cultures is called "Streaking for Isolation." We use the "four quadrant" method to achieve this isolation.

Inoculation of media is accomplished by one of three methods. The inoculation loop is used to inoculate liquid specimens such as sputum and body fluids. A small amount of the liquid will form a film over the loop and this film is transferred to the media. The inoculation needle is primarily used to transfer colonies from one medium to another medium. This instrument is also used as a primary means of inoculating tubed media. The sterile swab is used as a means of directly collecting clinical specimens. The swab is rolled onto the medium to insure that the specimen is deposited on the surface.

After media have been inoculated, the streaking technique is performed to obtain isolated colonies. Streaking is a progressive dilution of the bacterial flora resulting in separation of cells. The separation of these cells will result in the production of isolated colonies after the media have been allowed to incubate.

The procedure used in this laboratory to obtain isolation is called the "four quadrant" method. The four quadrant method separates the bacteria by diluting the specimen over the entire surface by utilizing four different areas of the media to produce isolated colonies. We sterilize the inoculation instruments by incinerating them in the flame of a Bunsen burner. They must be allowed to cool before use, thereby preventing the destruction of bacteria by heat. The streaking manipulation is accomplished by keeping the streak lines close together. We also must be sure to spread (streak) the specimen over the entire surface of the medium.

There are many pitfalls in the streaking technique. Some of the more common errors are cutting into the surface of the media with the inoculation loop and improper utilization of the media surface. When the streak lines are too far apart the surface of the media is not properly utilized and poor isolation will result. Re-entry of a preceding quadrant may also result in poor isolation.

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*This instructor's manuscript supersedes IM 08-280-406, 015.

Restreaking is a process that is used when isolation has not been obtained. A small portion of the unisolated colonies is transferred to another plate and the streaking process is again attempted in order to obtain isolated colonies of the original culture. We sometimes use a procedure referred to as "subculturing" to obtain an additional amount of an organism in pure culture when there may be only a few isolated colonies on the original culture.

Different types of bacteria possess different observable characteristics when they grow on the surface of a medium. We commonly record these characteristics when observing bacterial growth on blood agar. Use of blood agar allows us to record one of the most important colony characteristics - hemolysis. Hemolysis is the effect bacterial colonies produce on red blood cells. We can observe three types of hemolysis on blood agar. Alpha hemolysis is a green zone surrounding the colonies in a halo-like manner. The red blood cells are left intact. Beta hemolysis is described as complete destruction of the red blood cells. There is a clear zone surrounding colonies that are beta hemolytic. Gamma hemolysis has little effect on the blood agar. The red blood cells are left intact, and there is no zone of destruction or discoloration surrounding the colonies.

We can also describe colonies according to their opacity. Some colonies are described as being clear or resembling a droplet of oil. They allow light to pass through freely. Some colonies are translucent. They are slightly cloudy and allow only a portion of the light to pass through them. Opaque colonies block out almost all light.

The descriptive term of size is also helpful in identifying bacterial colonies. The terms small, medium, and large are used to identify bacterial colonies but these terms are relevant primarily to the individual technician. In some instances this criteria may be the only one available to tell differences in bacterial growth. Obviously, size is not a distinct descriptive term because colonies of the same type of bacteria may differ in size.

Color is a descriptive term that may be used to aid us separate different types of colonies. Bacterial colonies usually develop one of three colors, ranging from white to gray to a yellow-orange pigment. Of all the criteria mentioned for distinguishing colony morphology, hemolysis on blood agar is probably the most definitive means of differentiating bacterial colonies.

Colony morphology on differential media will include any reaction surrounding the colony. On differential media, we look for a color change that is due to utilization of a metabolite, causing a change in the pH of the media. The pH change is revealed by a color indicator. For example, on mannitol salt agar, growing colonies may cause a pH indicator to turn yellow. The colonies may appear yellow; however, since this is a differential media, the descriptive term for the colonies would not be yellow pigmentation

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but would be "growth and fermentation." These descriptive terms will be taught later. It is important that we utilize gross colony morphology to help in identifying different types of colonies according to their consistency. Colony morphology may be used as an instrument to determine the success of isolation.

Subculturing technique is a process that we use to gain a greater number of pure colonies that are initially present in small numbers on an isolation medium. The procedure is performed by selecting an isolated colony and, using an inoculating needle, transferring it to another plate of medium that will support the growth of this colony. We then streak with the inoculating loop, which provides an additional amount of the original colonies to be used for additional staining or biochemical tests. Contamination is the most common error here and must be avoided. The most common cause of contamination is the transferral of some unwanted colonies from the original plate to the subculture. The subculture is incubated for 18 to 24 hours at 37°C under the same environmental conditions as the original culture. After incubation, the subculture is compared to the original culture to insure that the colonies are the same as the type of colony that we wish to reproduce. The subculture plate is also checked for extraneous bacterial contamination and for mixed culture.

All the techniques which were discussed and demonstrated must be mastered if bacteria are to be successfully isolated from clinical specimens and subsequently identified.

SFC J. A. Goode

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-407
LABORATORY SCIENCES DIVISION 038
MICROBIOLOGY BRANCH

Inoculation Techniques for Isolating and Demonstrating Colony Morphology - 2 hours

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES: All previous references

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: M 08-280-407-1 prior to class

PERSONNEL: Eight instructors, MOS 92B3H

INSTRUCTIONAL AIDS: Materials for practical exercise (see Annexes A and B); chalk, chalkboard, and eraser

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Practical exercise

I. INTRODUCTION (5 min)

A. Opening Statement: During this exercise you will be required to work with bacteria isolated from simulated specimens. This exercise is designed to give you practice in performing procedures routinely used for isolating and demonstrating colony morphology.

B. Objectives: Given bacterial specimens, and all necessary equipment inoculate media, streak for isolation and report morphology, as demonstrated in class.

C. Class Procedure and Lesson Tie-In

1. Application of information received during previous lectures and demonstrations.

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*This lesson plan supersedes LP 08-280-407, 01C

2. Practical exercise with various types of bacteria will be conducted for two hours on two consecutive days. Specific tasks assigned by instructor during each phase will lead to completion of the exercise by the final hour.

3. Cultures will be utilized in subsequent PE's.

II. APPLICATION (80 min)

A. Instructors' Activities

1. Divide the class into two-person teams.
2. Equipment and materials issued (Annex A)
3. Pure cultures prepared and issued (Annex B)
4. Daily instructions given
5. Previous day's activities reviewed
6. Questions answered
7. Corrections made
8. Laboratory safety maintained
9. Exercise classroom control

B. Students' Activities (see M 08-280-407-1)

1. Aseptic technique maintained
2. Practice streaking two uninoculated plates
3. Inoculate and streak blood agar plates
4. Colony morphology observed and recorded
5. Culture materials discarded
6. Clean up performed as directed
7. Equipment stored

C. Organization of Instructional Hours

1. First Day (40 min)(see Annex A)
    - a. Practice streaking
    - b. Inoculation of sheep blood agar
    - c. Cultures incubated
    - d. Specimens discarded
    - e. Clean up performed
2. Second Day (40 min)
    - a. Isolation attempt evaluated
    - b. Colony morphology studied and recorded

NOTE: Instructor leads entire class in the study of morphology in a step-by-step manner.

- c. Master recognition of hemolytic patterns.
- d. Clean up performed - hold plates to be used in later PE's

III. REVIEW (5 min)

A. Questions From Students

B. Review of Main Points

1. Inoculating and streaking techniques
2. Colony morphology

C. Closing Statement: Knowledge, however broad, of the theories and principles of clinical bacteriology is insufficient. You must develop the ability to apply your knowledge in a planned, orderly manner so that you can satisfactorily perform your duties as a medical laboratory technician.

INSTRUCTIONAL HOURS

Number of instructors required - - - 8

Primary hours 16

Total 16

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ANNEX A
INSTRUCTIONAL AIDS

Tools, Equipment, and Materials

A. Items Required

1. Burner and tubing (one per student)
2. Instrument kit (one per student)
3. Disinfectant
4. Paper towels
5. CO_2 Incubator
6. Incubator
7. Refrigerator
8. Autoclave buckets
9. Autoclave
10. Swab tubes
11. Masking tape
12. 40 hole racks
13. Wax markers

B. Additional Materials for First Day

1. Practice plates (2 per student)
2. Blood agar plates (5 per student)
3. Study set of organisms (see Annex B)

ANNEX B
INSTRUCTIONAL AIDS

Procedure for preparation of Practical Exercise

A. Materials

1. Ten bottles of Todd-Hewitt broth (50 ml/btl)
2. Stock cultures
    - a. Enterobacter species
    - b. Beta Streptococcus species, not Gp A
    - c. Streptococcus pneumoniae, Type 1
    - d. Staphylocccus aureus
    - e. Neisseria species
    - f. Gamma Streptococcus species, Gp D
    - g. Beta Streptococcus species, Gp A
    - h. Alpha Streptococcus species
    - i. Staphylococcus epidermidis
    - j. Gamma Streptococcus species, not Gp D
3. Forty-hole swab tube rack (one per two students)
4. Swab tubes (5 per student)
5. Felt tip marker
6. Masking tape

B. Preparation procedure: On the day prior to the PE, inoculate one Todd-Hewitt broth with each of the ten listed organisms. The Streptococcus pneumoniae and the Neisseria should be inoculated to SBA and a broth make from the colony growth on the day of the PE.

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C. Distribution procedure

1. Label Todd-Hewitt broths as follows:

   Mixture # 1. Alpha *Streptococcus* species
   2. Beta *Streptococcus* species, not Gp A
   3. Gamma *Streptococcus* species, not Gp D
   4. *Staphylococcus aureus*
   5. *Enterobacter* species
   6. *Streptococcus pneumoniae*, Type 1
   7. Beta *Streptococcus* species, Gp A
   8. Gamma *Streptococcus* species, Gp D
   9. *Staphylococcus epidermidis*
   10. *Neisseria* species

2. Dip swabs into appropriate cultures and distribute in accordance with Protocol 1. (One rack per two students)

   A class of 40 students needs 20 swab tubes of each mixture.

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PROTOCOL 1

Mixtures to Use in Series

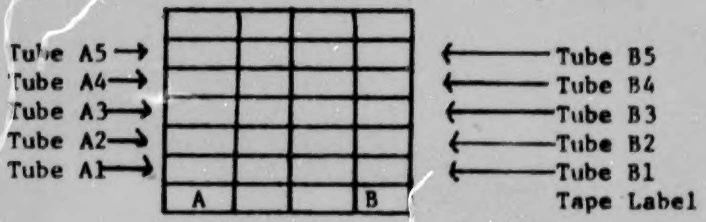
Series A

Mixture #	Tube #
1	1 A
2	2 A
3	3 A
4	4 A
5	5 A

Series B

Mixture #	Tube #
6	1 B
7	2 B
8	3 B
9	4 B
10	5 B

Diagram of Rack


Tube A5
Tube A4
Tube A3
Tube A2
Tube A1
A
B
Tube B5
Tube B4
Tube B3
Tube B2
Tube B1
Tape Label

NOTE: One rack arranged in this manner is needed for every two students. Care must be taken to arrange organisms exactly as indicated so that they will match the students instruction sheet (M 08-280-407-1).

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-407-1
LABORATORY SCIENCE DIVISION 038
MICROBIOLOGY BRANCH

Procedure for Streaking, Gram Stain, Morphology, and Biochemical Test: Practical Exercise

FIRST DAY

A. Streaking for Isolation (40 min)

1. Obtain two practice agar plates and use them to practice the "four quadrant" streaking technique.
   After you have completed the practice, ask an instructor to evaluate your work.

2. The class will then be divided into groups of two. Each group will be issued ten cultures in one rack as indicated in Figure 1 below. One student should use the swabs labeled A and the other student should use the swabs labeled B.

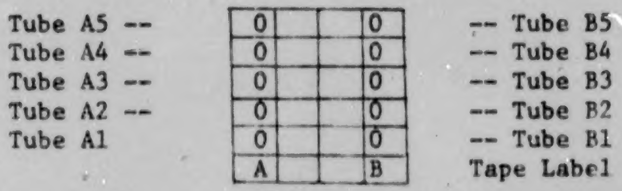


Figure 1

3. Obtain 5 sheep blood agar plates and label one for each culture on the back of the plate using your wax pencil. The label should include your student number date, and culture number (e.g., A-1 and A-2).

4. Using proper aseptic technique, inoculate the labeled media by rolling the swab on the surface of the appropriately labeled petri dish. Using your wire loop, streak all SBA plates for isolation using the four quadrant method.

5. Stack inverted SBA plates in the canisters and incubate as directed by the instructor.

6. Clean up as directed.

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*This mimeo supersedes M 08-280-407-1, 026

SECOND DAY (135 min)

A. Streaking

1. Remove cultures from incubator and observe the colony morphology of each organism as it appears on sheep blood agar.

2. Check isolation; are colonies isolated?

3. Record colony size, color, and hemolytic pattern for all ten organisms in your group.

B. Gram Stain

1. The class will remain in the same groups of two. Keep your plates from the streaking practical exercise out for use in the gram stain practical exercise.

2. Label three slides each with the following culture numbers: A-4, A-5, and B-5.

3. Prepare three smears of each of the above specified cultures by placing a loopful of water onto a clean, labeled glass slide. Take a sterile needle and touch one or two colonies with the tip of the needle. Make an emulsion on the slide and spread it out evenly to the size of a dime. Let it air dry and then heat-fix it.

4. Stain one slide of each set of slides that you have prepared (one slide of A-4, one slide of A-5, and one slide of B-5) Do Not stain the other slides at this time.

5. Compare your results with those that the instructor lists on the chalkborad.

6. If your results are the same as the instructors, make smears from the remaining cultures in your group of cultures and stain them.

7. If your results do not agree with the instructor's, stain another set of the slides you prepared earlier, using techniques that will rectify the errors.

8. After work is completed, place all plates in metal canisters provided and refrigerate as directed.

9. Put away scopes properly, discard slides and perform general clean-up as directed.

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THIRD DAY (90 min)

A. Biochemical Tests

1. Remove the inoculated sheep blood agar plates from the refrigerator (these are the plates you used in the Streaking and Gram Stain P. E.).

2. Do only the tests that are indicated for your specimen numbers. However, observe the test results obtained by other members in your group.

3. Complete the following test on indicated specimens. Label tubes or plates the same as the original specimen.

Specimen	Test
A-1	Catalase; and using one half a sheep blood agar do an optochin disc susceptibility.
A-2	Using the other half of the sheep blood agar, do a bacitracin susceptibility disc test.
A-3	Inoculate bile esculin azide (BEA) tube.
A-4	Catalase and coagulase.
A-5	Perform oxidase test.
B-1	Catalase; and using one half of a sheep blood agar plate do optochin disc susceptibility.
B-2	Catalase; and using the other half of the sheep blood agar plate do a bacitracin susceptibility disc test.
B-3	Inoculate bile esculin azide (BEA) tube.
B-4	Catalase and coagulase.
B-5	Perform oxidase test.

4. Record results of catalase and oxidase tests (page 5).

5. Incubate inoculated tests as directed by the instructor.

6. Refrigerate the plates from which the biochemical tests were inoculated.

7. Clean up as directed.

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FOURTH DAY

A. Biochemicals

1. Remove inoculated tests from the incubator and record the results of these tests (page 5).
2. Observe and record the test results that the other person in your group has obtained.
3. Compare your results to those that the instructor indicates you should have. If you have noted any discrepancies, discuss them with an instructor.
4. Clean up as directed.

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Spec#	Colony morphology sheep blood agar (size, color, hemolysis)	Gram stain (shape, arrangement reaction)	Biochemical Tests					
			Catalase	Coagulase	Oxidase	BEA	Optochin	Bactracin
A-1								
A-2								
A-3								
A-4								
A-5								
B-1								
B-2								
B-3								
B-4								
B-5								

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-408
LABORATORY SCIENCES DIVISION 048
MICROBIOLOGY BRANCH

Gram Stain Procedures - 2 hours

COURSES PRESENTED TO: 311-92B10 Medical Laboratory Procedures, Basic

PLACE CLASSROOM: Classroom

REFERENCES:

Baily, W. R. and Scott, E. G. Diagnostic Microbiology. 4th ed St. Louis; C. V. Mosby Company, 1974, chap 2 and 3

Lennette, E. H. et al (ed) Manual of Clinical Microbiology. 2nd ed. Washington: American Society for Microbiology. 1974, chap 6 & 96.

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: M 08-280-408-1 issued prior to class

PERSONNEL: One primary instructor (MOS 92B30)

INSTRUCTIONAL AIDS: Chalk, chalkboard, and eraser; TV No. 187 (see Annex A); television receivers, MP 08-280-408-1 through 19 (see Annex B);

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

I. INTRODUCTION (5 min)

A. Opening Statement: The most widely used staining technique in the bacteriology laboratory is the Gram stain. Properly performed, this technique provides the technician with vital information for the identification of bacteria.

B. Objective

1. Given a list of steps, select the steps in the proper sequence for performing a Gram stain.
2. Given a description of a Gram stained specimen, select the correct gram morphology to include reaction, shape, and arrangement.

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*This lesson plan supersedes LP 08-280-425, 036

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3. Given a situation select the proper technique for storage handling and operation of the microscope.

C. Class Procedure and Lesson Tie-in: Students will utilize the techniques and procedures covered in this lecture during subsequent practical exercises.

II. EXPLANATION (70 min)

A. The Gram Stain

NOTE: Show placards as applicable (see Annex B)

1. Purpose

a. Used for the primary differentiation of bacteria

b. Gives bacteria a color for ease of microscopic observation

2. Smear preparation

a. From plating medium

(1) Label slice

(2) Add a loop-full of water to the middle of the slide

(3) Use a needle to pick a colony from the plate

(4) Emulsify the colony in the water

(5) Let the smear air dry completely

(6) Heat fix

b. From broth medium

(1) Label slide

(2) Mix the broth

(3) Place a loopfull of broth on the middle of the slide

(4) Spread broth to the size of a dime.

(5) Let the smear air dry completely

(6) Heat fix

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QUESTION: When are Gram stain smears heat fixed?

ANSWER: Only after the smear has completely air dried.

3. Staining procedure
    a. Crystal violet (blue)
        (1) Flood the slide for one minute
        (2) Rinse the slide with cold tap water
        (3) Touch the end of the slide to a paper towel to remove any excess water
    b. Gram's iodine (brown)
        (1) Flood the slide for one minute
        (2) Rinse the slide with cold tape water
    c. Decolorizer (clear)
        (1) Hold the slide at a 45° angle
        (2) Apply the decolorizer to the frosted end of the slide
        (3) Allow the decolorizer to run down over the entire smear
        (4) Rinse with cold tap water
    d. Safranin (red)
        (1) Flood the slide for 30 seconds
        (2) Rinse with cold tap water
    e. Blot the smear dry with a paper towel
4. Trouble-shooting the Gram stain
    a. Overdecolorized smear
        (1) Decrease the time and amount of decolorizer
        (2) Insure that the Gram's iodine is not diluted with water from the previous washing

b. Underdecolorized smear

  (1) Slightly increase time and amount of decolorizer

  (2) Make sure that decolorizer reaches all areas of the smear

NOTE: Thick smears generally take longer to decolorize, while thin smears decolorize more rapidly.

B. Interpretation of Gram Morphology

1. Gram reaction

   a. Blue to purple bacteria, report as gram positive

   b. Red to pink bacteria, report as gram negative

2. Shape

   a. Spherical cells, report as cocci

   b. Cylindrical cells, report as bacilli

   c. Elongated spheres, report as coccobacilli

3. Arrangement

   a. Clusters of cells, report in clusters

   b. Chains of cells, report in chains

   c. Pairs of cells, report in pairs

   d. No predominant arrangement, report NPA

4. Examples and reporting format

   a. All reported gram morphologies must include gram reaction, shape, and arrangement

   b. Microscopic observation

      (1) Blue bacteria

      (2) Spherical cells

      (3) Generally clumped together

   c. Gram stain report--gram positive cocci in clusters

NOTE: Other combinations of reactions, shapes and arrangements may be given at this time

QUESTION: What three items must be included when reporting gram morphology?

ANSWER: Gram reaction, shape, and arrangement of the bacteria.

C. Videotape (see Annex A)

1. Introduction

a. The Gram Stain (9.17 minutes)

b. Points to be observed

(1) Preparation of slides

(2) Application of staining reagents

(3) Interpretation of results

2. Showing of Videotape (TV NO. 187)

D. Effective use of the Microscope

1. Handling of the microscope

a. Use only your assigned microscope

b. Transport utilizing both hands to carry the microscope

(1) Grasp the arm of the microscope

(2) Support the base

(3) Cradle the rheostat

(4) Always hold the microscope in an upright position

c. Do not remove oculars

2. Operation of the microscope

a. Light adjustment

(1) Select the 4.5 setting on the rheostat

(2) Select the low power objective

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(3) Adjust the oculars for interpupillary comfort

(4) Place the neutral density filter lever on dim.

(5) Fully open the field diaphragm lever

(6) Fully open the condenser aperture diaphragm lever

(7) Insure the swing in condenser is out of the light path

(8) Place a slide on the micrscope stage with the smear side up

(9) Raise the Abbe condenser to within a paper thickness of the slide.

b. Low power observation

(1) Clean all exposed lenses with lens paper only

(2) Lower the coarse and fine adjustments

(3) Look through both eyepieces

(4) Focus using first the coarse, then the fine adjustment

QUESTION: Why is lens paper only used to clean the exposed lenses?

ANSWER: Lens paper is the only cleaning paper that will not scratch the lenses.

c. High dry observation

(1) Place the neutral density filter on bright

(2) Select the high dry objective

(3) Focus using the fine adjustment only

(4) Adjust the field diaphragm lever and the condenser aperture diaphragm lever for maximum contrast

d. Oil immersion observation

(1) Focus the smear using the low power objective

NOTE: See low power observation procedure.

(2) Add one drop of immersion oil to the middle of the smear

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(3) Place the neutral density filter on bright

(4) Select the oil immersion objective

(5) Focus using the fine adjustment only

(6) Adjust the field diaphragm lever and condenser aperture lever for maximum contrast

QUESTION: Why is the fine adjustment only used to achieve sharp focus when using high dry or oil immersion objective?

ANSWER: To insure the objectives do not come into contact with the slide, which could result in scratching the objectives.

3. Sources of error prohibiting focus

a. Slide is placed on the microscope smear side down

b. The light adjustment settings are incorrect

c. Dirty microscope lenses

d. Improper use of the fine and coarse adjustments

NOTE: When you are not able to focus the microscope, correct any errors, repeat the light adjustment and start over using the low power observation procedures.

4. Storage of the microscope

a. Select low power

b. Clean all oil and stain off of the microscope

NOTE: Use lens paper only on the lenses.

c. Return the microscope to its numbered position in the cabinet.

F. Questions from Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Gram stain

2. Interpretation of gram morphology

3. Storage, handling, and operation of the microscope

P. Closing Statement: The gram stain technique is a diagnostic procedure of the bacteriology laboratory which can be of immense value when properly performed. During the ensuing weeks you will have the opportunity to practice this procedure on bacteria whose Gram staining properties are known.

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ANNEX A
INSTRUCTIONAL AIDS

Videotape

TV No. 187, 9.17 minutes. The Gram Stain.

This videotape begins with methods of preparing bacterial smears for staining on microscopic slides. The sources of bacteria demonstrated are a broth culture, an isolated colony from solid medium, and a clinical specimen such as sputum. Emphasis is placed on putting no more than one smear on a slide allowing the slide to air dry, and gently heat fixing the slide prior to staining. The actual staining technique is demonstrated on one of the prepared slides. The name and function of each reagent as well as sources of error are discussed during the staining procedure. Photomicrographs of properly Gram stained bacteria are shown while the interpretation of the Gram stain is explained.

NOTE: This videotape is shown in part IID.

ANNEX B
INSTRUCTIONAL AIDS

Magnetic Placards

MP 08-280-408-1	Gram Stain Procedure
MP 08-280-408-2	Storage of Microscope
MP 08-280-408-3	Microscopic Morphology
MP 08-280-408-4	Gram Stain Technique
MP 08-280-408-5	Purpose of Staining
MP 08-280-408-6	Procedure of Staining
MP 08-280-408-7	Smear Preparation
MP 08-280-408-8	Label Slide
MP 08-280-408-9	Apply Specimen
MP 08-280-408-10	Air Dry
MP 08-280-408-11	Heat fix
MP 08-280-408-12	Staining
MP 08-280-408-13	Crystal Violet
MP 08-280-408-14	Gram's Iodine
MP 08-280-408-15	Decolorizer
MP 08-280-408-16	Safranin
MP 08-280-408-17	Tapwater
MP 08-280-408-18	Handling of Microscope
MP 08-280-408-19	Operation of Microscope

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LABORATORY SCIENCES DIVISION 048
MICROBIOLOGY BRANCH

Gram Stain Procedure

The most widely used staining technique in the bacteriology laboratory is the Gram stain. Properly performed this technique provides the technician with vital information for the identification of bacteria.

The basis for identifying isolated bacteria begins with morphological characteristics. Bacteria are unicellular. Most bacteria have a cell wall, which is a cellulose-like structure that gives shape and rigidity to the cell. Just under the cell wall is the cytoplasmic membrane. It is semipermeable and governs osmotic activity, whereby nutrients diffuse in and wastes are removed. The cell membrane surrounds the cytoplasm and is the site of metabolism. In conjuction with ribonucleic acid, all chemical reactions occur within it to sustain life of the bacterium. Cytoplasm is a colloidal composition of amino acids, water, salts, and other compounds plus inclusion bodies of granules and vacuoles. These granules consist of organic and inorganic compounds and serve as a means of food storage or as waste receptacles. The cellular nucleus is diffuse and under the optical microscope does not appear as a discrete body, although recently the electron microscope has shown a distinct nucleus dividing evenly between the daughter and mother cell during binary fission. It is composed of desoxyribonucleic acid and a primary function is cellular replication. A mucoid, gelatinous substance attached to the outer surface of the cell wall of many bacteria is the capsule (slime layer). This structure affords protection to a bacterium by resisting phagocytosis and associated virulence. Some bacteria produce spores (endospores) which are hard, dense, dehydrated structures formed within the cell. The spore contains all essential cell material; it is a resting cell, metabolically inactive. The function of a spore is to resist unfavorable environmental conditions. One vegetative cell produces one spore; when growth conditions become favorable, one spore produces one vegetative cell. The shape and location of spores aid in identification of the organism. Some bacteria have one or more thin hairlike strands of protein extending from the cytoplasm to the external area of the cell. This structure is a flagellum. Bacteria which have flagella are motile since flagella are organs of locomotion. The possession of a flagellum by a bacterium, including the number and location, is important in identification schemes. Special techniques must be employed to observe the various structures of a bacterial cell.

The microscopic morphology; i.e., the shape, arrangement, and size of bacterial cells, is a valuable diagnostic tool. There are three basic shapes: the sphere or coccus; the rod or bacillus; and the corkscrew or spirochete. Coccobacillus is an intermediate form. Some bacterial cells have a pleomorphic shape. The arrangement of bacterial cells is a function of reproduction. Bacteria appear as single cells, in pairs, in groups of four, in clusters, and in chains. The predominant arrangement is specific for

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*This instructors manuscript supersedes IM 08-280-408, 036 a given bacterium.

The purpose of the Gram stain is twofold: first, it demonstrates the Gram morphology of bacteria which includes Gram stain reaction, shape of cells, and arrangement of cells. These three criteria must be met in order to properly report Gram morphology. Secondly, the Gram stain is a differential stain in that it separates bacteria into two different groups according to staining characteristics. Blue-purple staining bacteria are termed gram-positive while pink-red staining bacteria are termed gram-negative. The Gram stain is an important initial step in the identification of unknown bacteria and serves as a guide in selection of proper media which enhances recovery of bacteria in the submitted specimen.

Sources of bacteria to be stained include broth cultures, isolated colonies from solid media, and clinical specimens such as sputum. The first step in performing an accurate Gram stain is to prepare a bacterial smear on a microscope slide. Before preparing the smear, the slide must be properly labeled. Using a diamond tip pencil label the slide and prepare the smear on the same side. If a wax pencil is used, label the bottom side of the slide to prevent the label from being washed off during staining. If the organisms are grown in broth, gently shake the tube to suspend all growth. Place a loopful of the culture on a properly labeled, clean, glass slide. Spread the liquid evenly on the slide and allow the smear to air dry. Organisms grown on a solid medium are prepared by first placing a loopful of tap water on a clean, labeled slide. Pick up a small amount of the colony to be stained with a sterile needle, and emulsify the mixture by spreading it into a thin even film. When performing a Gram stain directly on material from a clinical specimen such as sputum, obtain a portion of the specimen with a sterile swab, and roll it over the surface of the slide. This results in an even smear suitable for staining. Allow all smears to air dry. Do not attempt to hasten the drying process by heating the slide. Excess heating results in distortion of bacterial cell walls, altering the shape.

After the smear has air dried, it is heat-fixed by rapidly passing the slide, smear side up, through a burner flame three to four times. Heat-fixing causes the bacteria to adhere to the slide and prevents them from being washed off during the staining procedure.

The smear is now ready to be Gram stained. Place the slide on a staining rack, smear side up. Flood the entire smear with crystal violet, the primary stain, and allow it to remain on the slide for at least one minute. Crystal violet imparts a blue-purple color to see bacteria. After one minute, tilt the slide to allow the crystal violet to run off and rinse with tap H_2O. Flood the smear with Gram's iodine. This reagent acts as a mordant which fixes the primary stain in gram-positive cells. As in step one, both gram-positive and gram-negative bacteria are stained blue-purple at this point.

Allow the Gram's iodine to remain on the slide for at least one minute and again tilt the slide, allowing the reagent to run off and rinse. The next step, decolorization, is the most crucial step in the staining procedure. Decolorize with a mixture of equal volumes of acetone and 95 percent ethanol or straight 95 percent ethanol. Over-decolorization will yield false gram-negative results, while under-decolorization produces false gram-positive results. Decolorization is accomplished by removing excess and unbound crystal violet from the smear by running a solution of the decolorizer down one end of the slide and over the smear for three to five seconds. However, when color can no longer be seen breaking from the smear, regardless of time, immediately flood the smear with safranin to stop the action of the decolorizer. Before the addition of safranin, gram-positive organisms are stained blue-purple and gram-negative organisms are colorless. Allow the safranin, the counterstain, to remain on the slide for at least thirty seconds. Rinse with tap water.

The slide is air dried or blotted dry with bibulous paper. The smear can be observed using the oil immersion lens of the microscope. When interpreting the results of a Gram stain, organism with appear blue-purple are termed gram-positive, organisms staining pink-red are termed gram-negative.

The stains and reagents used are prepared according to the directions found in TM 8-227-5, Attachment I. After a new set has been prepared, check the quality by using the new reagents to stain known gram-positive and known gram-negative bacteria. Check the results of the staining procedure microscopically. Quality control testing should be performed with all new lots of reagents to insure validity of reactions.

The Gram stain technique is a diagnostic procedure of the clinical bacteriology laboratory which can be of immense value when properly performed. During the following periods you will have the opportunity to practice this procedure on bacteria whose Gram staining properties are known.

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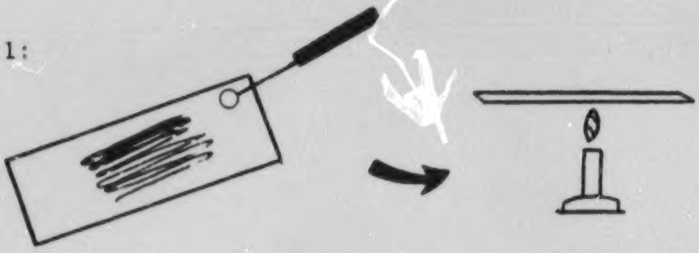
ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-408-1
LABORATORY SCIENCES DIVISION Oц8
MICROBIOLOGY BRANCH

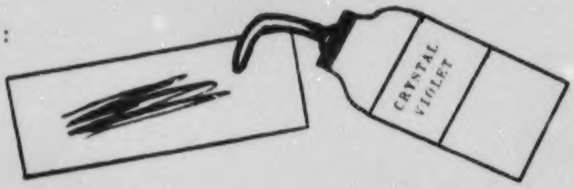
Gram Stain Procedure

Step No 1:



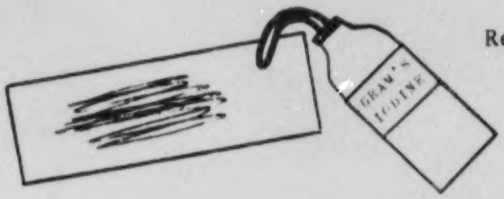
Prepare smear
AIR DRY
Gently heat fix

Step No 2:



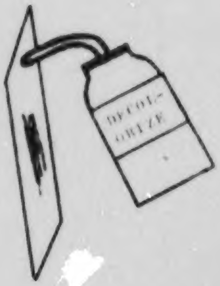
Flood slide with crystal violet.
1 MINUTE
Tip Slide, run off crystal violet.
Rinse with gentle flow of water.

Step No 3:



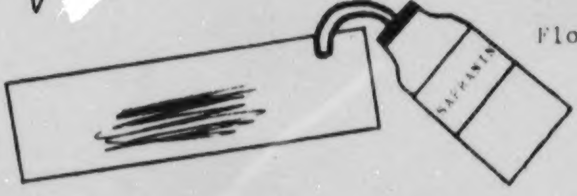
Replace crystal violet with Gram's Iodine.
1 MINUTE
Tip slide, run off Gram's Iodine.
Rinse with gentle flow of water.

Step No 4:



Decolorize with 50-50 acetone-ethyl alcohol or 95 % ethyl alcohol
BE VERY CAREFUL! **1 to 10 seconds only**
(By holding slide at 45° angle, you are able to rapidly stop decolorization by addition of safranin) or by rinsing with a gentle flow of water.

Step No 5:



Flood slide with safranin.
30 Seconds

Step No 6: RINSE WITH TAPWATER AND GENTLY BLOT DRY.

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*This mimeo supersedes M 08-280-408-2, Oц6

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M 08-280-408-1
048

Gram Morphology

The Gram morphology report consits of three descriptive areas, the Gram reaction, the shape, and the arrangement of the stained bacteria. All three must be included in a proper and complete report.

A. Gram Reaction

1. Cells that stain blue to purple are termed Gram positive
2. Cells that stain pink to red are termed Gram negative

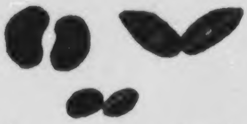
B. Shapes

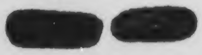
1. Cells that are ball-or sphere-shaped are classified as cocci
2. Cells that are rod-shaped are classified as bacilli
3. Cells that are spiral-or spring-shaped are clssified as spirochetes
4. Cells that are elongated spheres are classified as coccobacilli

C. Arrangement

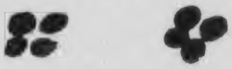
1. The arrangement of bacterial cells is determined by the formation that they demonstrate on the Gram stain slide

   a. Bacteria in groups of two


(cocci in pairs)


(bacilli in pairs)

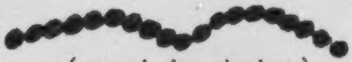
   b. Bacteria in groups of four

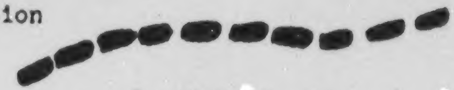

(cocci in tetrads)

   c. Bacteria in grape-like clusters or clumps

(cocci in clusters)

   d. Bacteria in chain like formation


(cocci in chains)


(bacilli in chains)

2. Bacilli usually have no characteristic arrangement. They may occur as single cells, pairs, or chains. If no predominant arrangement is demonstrated by bacilli report their arrangement as "NPA".

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
MICROBIOLOGY BRANCH

SP4 M. Ciaccio
*LP 08-280-409
048

Gram Stain Practical Exercise (4 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic)

PLACE: Classroom

REFERENCES: All previous references

STUDY ASSIGNMENT: Review M 08-280-407-1 prior to class

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: See Annex A and B

PERSONNEL: Eight instructors (MOS 92B3H)

INSTRUCTIONAL AIDS: None

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Practical exercise, -1

I. INTRODUCTION (5 min)

A. Opening Statement: Frequently you may be asked to submit a presumptive report on the bacteria present in a clinical specimen. The basis of your report will be the results you obtain by performing a Gram stain. You must also be able to perform and interpret a Gram stain on organisms isolated by cultural techniques in order to identify the bacteria recovered. Your examination and report can only be as accurate as your ability to properly perform the Gram stain technique.

B. Objective

Given bacterial specimens, and all necessary materials, prepare, stain and interpret smears and report Gram Morphology of smears, as demonstrated in class.

C. Class Procedure and Lesson Tie-in

1. Application of information presented in previous lecture.

2. Future Gram staining exercises conducted during the duration of the course.

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*This lesson plan superseded LP 08-280-409-055

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II. APPLICATION (140 min)

A. First Two Hours of PE

NOTE: The first two hours of this PE follows PE 08-280-407

1. Day prior to PE

a. Instructors' activities

(1) Inoculate Gram stain controls (see Annex A)

(2) Quality control Gram stain reagents and organism staining reactions.

(3) Insure that sufficient tools and equipment are available for the following days PE (see Annex B)

2. Day of PE

a. Instructors' activities

(1) Prior to PE

(a) Check inoculated student plating media from PE 08-280-407 for organism content and contamination

(b) Check instructor inoculated controls for organism content and contamination

(2) During PE

(a) Issue tools and equipment (see Annex B)

(b) Explain days activities

NOTE: Demonstrate smear preparation and gram stain technique.

(c) Instruct students to obtain plating media from PE 08-280-407

(d) Issue Gram stain control plating media.

(e) Maintain order and safety

(f) Critique student results

(g) Correct Gram stain technique errors

(h) Answer questions

(i) Supervise clean-up

(j) Refrigerate culture material

b. Students' activities

(1) Area prepared for PE

(2) Equipment obtained

(3) Prepare slide--source of organisms indicated by instructor (see M 08-280-407-1 for instructions)

(4) Gram stain performed on organisms from plating media

(5) Results interpreted

(6) Results shown to instructor

(7) Equipment stored

(8) Clean-up performed

B. Hours Three and Four of PE

NOTE: Hours three and four of this PE follows PE 08-280-416

1. Day prior to PE

a. Instructors' activities

(1) Inoculate Gram stain controls (see Annex A)

(2) Inoculate required cultures (see Annex C)

(3) Insure that sufficient tools and equipment are available for the following days PE (see Annex B)

(4) Quality control Gram stain reagents and organism staining reactions

2. During PE

a. Instructors' activities

(1) Prior to PE

(a) Check instructor inoculated plates for organism content and contamination

(b) Mix and dispense broth cultures (see Annex C)

(c) Check instructor inoculated controls for organism content and contamination

(2) During PE

(a) Issue tools and equipment (see Annex B)

(b) Issue inoculated and labeled plates (see Annex D)

(c) Issue Gram stain control plating media (see Annex A)

(d) Explain days' activities

(e) Maintain order and safety

(f) Critique student results

(g) Correct gram stain technique errors

(h) Answer questions

(i) Supervise clean-up

b. Students activities

(1) Area prepared for PE

(2) Equipment obtained

(3) Prepared slides--source of organisms indicated by instructor

(a) Prepare two slides from each issue labeled plating media (see Annex C)

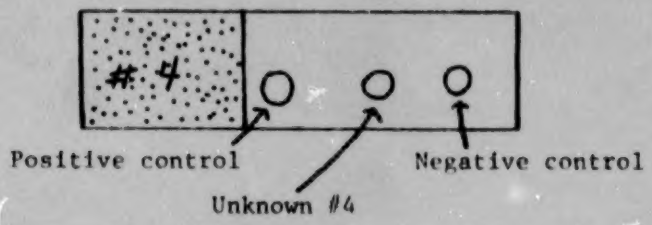
(b) Prepare two slides from the issue broth culture (see Annex C)

(c) Each unknown smear should be flanked by a positive and negative control

(d) Example

1 Unknown plate number 4

2 Diagram of slide



(4) Gram stain performed on organisms from plating media and broths

(5) Results interpreted

(6) Results shown to instructor

(7) Equipment stored

(8) Clean-up performed

III. REVIEW (15 min)

A. Correct Interpretations Given to Students

B. Questions from Students

C. Review of Main Points

1. Gram stain technique

2. Microscopic examination of slides

3. Recording results

D. Closing Statement: The staining technique you have performed during this period is one of the most important and most often performed in the bacteriology laboratory. Thorough mastery of the techniques of the Gram stain and interpretation of results is required to properly carry out your duties in the bacteriology laboratory.

ANNEX A

Inoculation of Gram Stain Controls

A. Inoculate Staphylococcus aureus to Mueller Hinton plating medium and streak for confluent growth.

B. Inoculate Enterobacter species to Mueller Hinton plating medium and streak for confluent growth.

C. Incubate Mueller Hinton plates 18-24 hours at 37 C the day prior to the Gram stain practical exercise.

D. Inoculate one set of Gram stain controls (one MHA plate with the gram positive and one plate with the gram negative organism) per four students.

ANNEX B
INSTRUCTIONAL AIDS

Tools and Equipment

1.	Autoclave, laboratory	1 per class
2.	Incubator, large	1 per class
3.	Microscope, binocular, complete	1 per student
4.	Loop and needle, inoculating	1 per student
5.	Pencil, wax	1 per student
6.	Gram stain reagents (set)	1 per sink (7)
7.	Gram stain controls	1 set per 4 students
8.	Disinfectant	1 gal per class
9.	Detergent, surgical	1 gal per class
10.	Test tube racks, 40-hole	1 per two students
11.	Laboratory burner	1 per student
12.	Decontamination buckets	3 per class
13.	Paper towels	7 pkg per class
14.	Microscopic slides	1 box per student
15.	Immersion oil	1 bottle per student
16.	Staining rack and tray (complete)	1 per sink (7)
17.	Masking tape	1 roll per class

ANNEX C

Inoculation and Incubation of Required Cultures

A. Inoculation of Plating Media

1. Organisms utilized and media required

NOTE: Inoculate each of the following organisms to the indicated plating media. Inoculate one plate of each organism for each four students and streak for confluent growth.

a. Staphylococcus aureus-Mueller Hinton agar

b. Bacillus species-Mueller Hinton agar

c. Enterobacter species-Mueller Hinton agar

d. Neisseria species-sheep blood agar

2. Labeling and incubation of inoculated plating media.

a. Labeling

(1) Label all Staphylococcus aureus plates #1

(2) Label all Bacillus species plates #2

(3) Label all Enterobacter species plates #3

(4) Label all Neisseria species plates #4

b. Incubate all plates in the CO_2 incubator for 18 to 24 hours at 37 C

B. Inoculation and Incubation of Broth Media

1. On the day prior to the PE inocu[illegible] Enterobacter species to a Todd-Hewitt broth

2. On the day prior to the PE inoculate Staphylococcus aureus to a Todd-Hewitt broth

3. Incubate both broths in the walk-in incubator for 18 to 24 hours at 37 C

4. On the day of the PE mix two parts of the Enterobacter species broth with one part of the Staphylococcus aureus broth

5. Dispense three ml per tube in 13 X 100 mm test tubes with caps

ANNEX D

Distribution of Incubated Cultures

A. Each group of four students will receive one set of the following cultured plating media:

1. One plate labeled 1
2. One plate labeled 2
3. One plate labeled 3
4. One plate labeled 4

B. Each group of four students will receive one 13 X 100 mm test tube containing the mixed broth culture.

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SSG Gerald B. Howe

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-410
LABORATORY SCIENCES DIVISION 048
MICROBIOLOGY BRANCH

Biochemical Testing Procedures (2 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic)

PLACE: Classroom

REFERENCES:

GR 09-280-456-1
Bailey, W. R. and Scott, E. G. Diagnostic Microbiology. 4th ed. St. Louis; The V.C. Mosby Co. 1974, chap 39, 40, & 41.
Lennette, E. H. and others (ed). Manual of Clinical Microbiology. 2nd ed. Washington; American Society for Microbiology, 1974, chap 95 & 96.

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: GR 09-280-456-1 issued prior to class

PERSONNEL: One instructor, MOS 92B3H

INSTRUCTIONAL AIDS: Chalk, magnetic chalkboard, and eraser; 35 mm Carousel projector and screen, S 08-280-410-1 thru -8 (see Annex A); TV No. 163 and recievers (see Annex B); equipment for demonstration (see Annex C and D); magnetic placards 1 thru 11 (see Annex E) chalkboard diagrams (see Annex F and G)

TROOP REQUIRMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Lecture and Demonstration

I. INTRODUCTION (5 min)

A. Opening Statement: Various types of bacteria have different and distinct biochemical properties. The medical laboratory technician can use these properties as a means of identifying unknown bacteria.

B. Objectives:

1. Given a description of biochemical test reactions, select the correct reporting terminology.

2. Given a list of cocci biochemical principles, select the

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*This Lesson Plan supersedes LP 08-280-410, 016

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principle that applies to specific biochemical tests.

C. Class Procedure and Lesson Tie-in

1. Following the class, a practical exercise in performing biochemical tests will be conducted.

2. Portions of the material presented will be tested by a written crucial examination later in the course

II. EXPLANATION (75 min)

NOTE: Refer to GR 09-280-456-1

A. Catalase Test

1. Purpose- to determine the presence of the enzyme catalase in certain bacteria.

2. Principle

    a. Catalase decomposes hydrogen peroxide

    b. $2H_2O_2 \dashrightarrow 2H_2O + O_2$

3. Reagent- 30 percent hydrogen peroxide (superoxol)

4. Procedure

    a. Transfer a colony to a clean glass slide

NOTE: Colony must be well isolated. Several colonies of the same morphology may be needed.

    b. Add one drop of 30% H_2O_2

    c. Observe for immediate bubbling

5. Interpretation

    a. Immediate bubbling-positive

    b. No bubbling-negative

NOTE: Show S 08-280-410-1

6. Cautions to avoid false positives

    a. Catalase enzyme is present in blood

(1) Do not perform test on surface of blood agar plates

(2) Do not transfer blood agar to the slide along with the colonies

b. Nichrome wire of inoculation needle may react with the hydrogen peroxide

c. Perform the steps in order

7. Application-aids in distinguishing Staphylococcus species from Streptococcus species

B. Coagulase Test

1. Purpose-to determine the presence of the enzyme coagulase in certain bacteria

2. Principle-coagulase causes plasma to clot

3. Reagent

a. Sterile rabbit plasma, citrated

b. 0.5 ml in sterile galss tube

c. Each batch must te tested prior to use

d. Human plasma not as good-it may contain inhibitory factors

4. Procedure

a. Transfer a colony to the reagent in tube using sterile cool needle

b. Incubate at 37 C for up to 24 hours

c. observe for clot

5. Interpretation

a. Clot formation-Positive

b. Not clot-negative

6. Application-Definative test for distinguishing pathogenic from non-pathogenic Staphylococcus species

NOTE: Show S 08-280-410-2

C. Oxidase Test

1. Purpose-to determine the presence of the enzyme oxidase in in certain bacteria

2. Principle-oxidase reagent + colonies producing indophenol oxidase yield characteristic color change

3. Reagent

    a. N,N,N',N' Tetramethyl-p-phenylenediamine with 0.1% ascorbic acid

    b. One percent solution in water

    c. Referred to as oxidase reagent

4. Procedure

    a. Select isolated colony for test

    b. Add one drop of reagent to colony on plate

    c. Tilt plate back and forth to allow colony to be exposed to air

    d. Observe for color change withing 30 seconds

5. Interpretation

    a. Color change in colony-positive

        (1) Dark blue to dark purple

        (2) Change must occur within 30 seconds

NOTE: Observing for longer than 30 seconds may lead to false positive result

    b. No color change within 30 seconds-negative

NOTE: Color change must accur in the colony for test to be positive. Color change in the medium is not signifigant.

6. Application-aids in identification of Neisseria species

NOTE: Show S 08-280-410-3 and -4

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D. Bile Esculin Azide Test (BEA)

1. Purpose-to determine if an organism has tne ability to hydrolyze esculin

2. Principle-bacterial enzymes hydrolyze esculin, forming a dark brown to black color

3. Ingredients

   a. Esculin-a protein

   b. Bile salts-inhibit most gram positive organisms

   c. Sodium azide-inhibits most gram negative organisms

4. Tubed medium

   a. Slant with butt

NOTE: Draw a diagram on chalkboard to illustrate this. Use diagram for chalkboard demonstration of inoculation procedure (see Annex G)

   b. Off-white color

5. Procedure

   a. Select well-isolated colony for test

   b. Transfer to tube

      (1) Use sterile cool needle

      (2) Stab butt straight to bottom center, fishtail streak slant

   c. Incubate-37 C for 18 to 24 hours

   d. Observe for color change

6. Interpretation

   a. Black or dark brown color throughout medium-positive

   b. No change-medium remains off white color-negative

7. Application-used in the grouping of Streptococcus species

NOTE: Show S 08-280-410-6

E. Optochin and Bacitracin Susceptibility Tests

1. Purpose-to determine the ability of an organism to grow in the presence of optochin or bacitracin

2. Principle-antibiotic diffuses into medium, inhibiting growth of susceptible organisms

3. Reagent-antibiotic impregnated paper disk

   a. Optochin (ethylhydrocupreine hydrochloride)

   b. Bacitracin

4. Procedure

   a. Select 3 to 5 identical well-isolated colonies for test

   b. Transfer to SBA plate, using sterile, cool needle

QUESTION: Why is sheep blood agar used for this test?

ANSWER: So that the hemolytic pattern of the organism may be observed.

   c. Streak for confluent growth

   d. Place disk on center of streaked area

      (1) Use forceps-alcohol dipped and flamed

      (2) Press disk gently with forceps to insure good cantact

   e. Incubate

      (1) 18 - 24 hours at 37 C

      (2) Increased CO2 - candle jar or CO_2 incubator

   f. Observe for zone of inhibition around disk

NOTE: Explain zone of inhibition and draw chalkboard diagram (see Annex F)

5. Interpretation

   a. Zone of inhibition around disk-susceptible (S)

   b. Growth of organisms up to disk-resistant (R)

NOTE: Stress that positive or negative are improper reports for this test

c. Criteria for valid test

(1) Pure culture

(2) Good, confluent growth

(3) Same organism as originally selected

QUESTION: How can you tell that it is the same organism?

ANSWER: Check the original plate. Colony morphology should be the same

NOTE: Show S 08-280-410-7 and -8

6. Application-these tests are used in the grouping of Streptococcus species

a. Optochin-used only on alpha hemolytic organisms. Differentiates alpha Streptococcus species from Streptococcus pneumoniae

b. Bacitracin-used only on beta hemolytic organisms. Differentiates beta Streptococcus species from Gp A Streptococcus species

NOTE: Stress that optochin disks are white, Bacitracin disks are brown

F. Sheep Blood Agar

1. Enriched medium

a. Used to grow fastidious organisms

b. Will also grow non-fastidious organisms

2. Ingredients

a. Basal medium

b. Sheep blood - 5% to 7 % sterile defibrinated

(1) Privides enrichment

(2) Indicator of hemolysis

QUESTION: What are the three types of hemolysis called, and what does each look like?

ANSWER: Alpha-green; beta-clear; gamma-no zone

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3. Preparation

    a. Sterilize basal medium by autoclaving

QUESTION: What autoclave settings should be used for this?

ANSWER: 121°C 15 lb/sq in, 15 min, slow exhaust

    b. Cool in water bath to 45-50°C

    c. Aseptically add sheep blood 50-70 ml/liter

4. Quality control

    a. Incubate all plates overnight at 37 C

    b. observe for contamination

    c. Refrigerate uncontaminated plates until use

NOTE: Packaging reduces water loss and increases shelf life

G. Videotape # 163 (14.43 min)

1. Introduction - Antibiotic Susceptibility Testing

2. Points to be observed

    a. Method of inoculation

    b. Disk placement

    c. Interpretation and reporting

3. Show videotape # 163

H. Questions from Students

III. SUMMARY (5 minutes)

A. Review of Main Points

1. Catalase test

2. Oxidase test

3. Coagulase test

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4. Bile esculin azide test

5. Optochin and Bacitracin tests

6. Sheep blood agar

B. Closing Statement: The use of a few simple biochemical tests in conjunction with colony morphology and microscopic morphology will allow the laboratory technician to identify a variety of pathogenic and non-pathogenic cocci.

ANNEX A
INSTRUCTIONAL AIDS

Slides

S 08-280-410-1	Slide of positive and negative catalase test
S 08-280-410-2	Slide of positive and negative coagulase test
S 08-280-410-3	Slide of a negative oxidase test
S 08-280-410-4	Slide of positive oxidase test on sheep blood agar
S 08-280-410-5	Slide of a positive and negative BEA test
S 08-280-410-6	Slide of the inoculation of a BEA tube
S 08-280-410-7	Slide of susceptible and resistant optochin test on sheep blood agar
S 08-280-410-8	Slide of susceptible and resistant bacitracin test on sheep blood agar

NOTE: These slides are shown throughout the lecture to demonstrate the subjects talked about.

ANNEX B
INSTRUCTIONAL AIDS

Videotape

TV No. 163, 14:43 minutes. Antibiotic Susceptibility Testing

This videotape is divided into two main parts: optochin and bacitracin susceptibility testing, and the Kirby-Bauer method of susceptibility testing. Optochin and bacitracin testing are similar in method and are treated together; they are utilized in the identification of certain bacteria. Sheep blood agar is the medium of choice for this method. Proper inoculation technique and placement of the discs are emphasized. Interpretation of the test results, including three criteria for a valid susceptibility test, and the proper reporting terminology are stressed. The Kirby-Bauer method is then considered. There is a short discussion on choosing the organism for testing, and the procedure for preparation of a standardized method of inoculation is stressed, so that the proper density of organisms and confluent growth will occur. Standards of concentration and the method of disc placement are the next considerations. Observation and interpretation of zones of inhibition, and proper reporting of test results are considered. Quality control is stressed. The tape ends with a brief summary of important points.

NOTE: Videotape shown in part II03

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ANNEX C
INSTRUCTIONAL AIDS

Demonstration Procedure

A. Catalase test

1. Transfer colony to glass slide
2. Add one drop 30 percent H_2O_2
3. Observe for immediate bubbling
4. Do positive and negative of each

NOTE: This demonstration is performed in part IIA3.

B. Coagulase Test

1. Transfer colony to media
2. Demonstrate use of proper equipment
3. Indicate incubation criteria

NOTE: This demonstration is performed in part IIB3.

C. Oxidase Test

1. Select colonies to be tested
2. Add one drop of reagent to colonies
3. Stress time limitation for test

NOTE: This demonstration is performed in part IID3.

D. BEA

1. Select proper BEA slant
2. Select colony for test

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3. Inoculate BEA
    a. Streak slant
    b. Stab butt
4. Indicate incubation criteria

NOTE: This demonstration is performed in part IIE4.

E. Susceptibility Tests

1. Optochin
2. Bacitracin
    a. Select colony for test
    b. Transfer
    c. Streak for confluent growth
    d. Place disk on inoculated area
    e. Indicate incubation criteria

NOTE: This demonstration is performed in part IIF5c.

ANNEX D
INSTRUCTIONAL AIDS

Equipment for Demonstration

1. Inoculating loop and needle
2. Touchmatic burner
3. Coagulase media 1 tube
4. H_2O_2 - 1 bt, 30 percent
5. Wax marker - one
6. One bottle of oxidase reagent
7. One vial of optochin sensitivity disks
8. One bottle of alcohol
9. One pair forceps
10. One vial of bacitracin sensitivity disks
11. One BEA tube
12. One sheep blood agar plate
13. One sheep blood agar plate beta Streptococcus sp
14. One sheep blood agar plate with Staphylococcus sp
15. One sheep blood agar plate with Neisseria sp

16. Videotrainer system will be utilized to show portions of prerecorded videotapes or to permit image magnification of the demonstration of each biochemical test.

ANNEX E
INSTRUCTIONAL AIDS

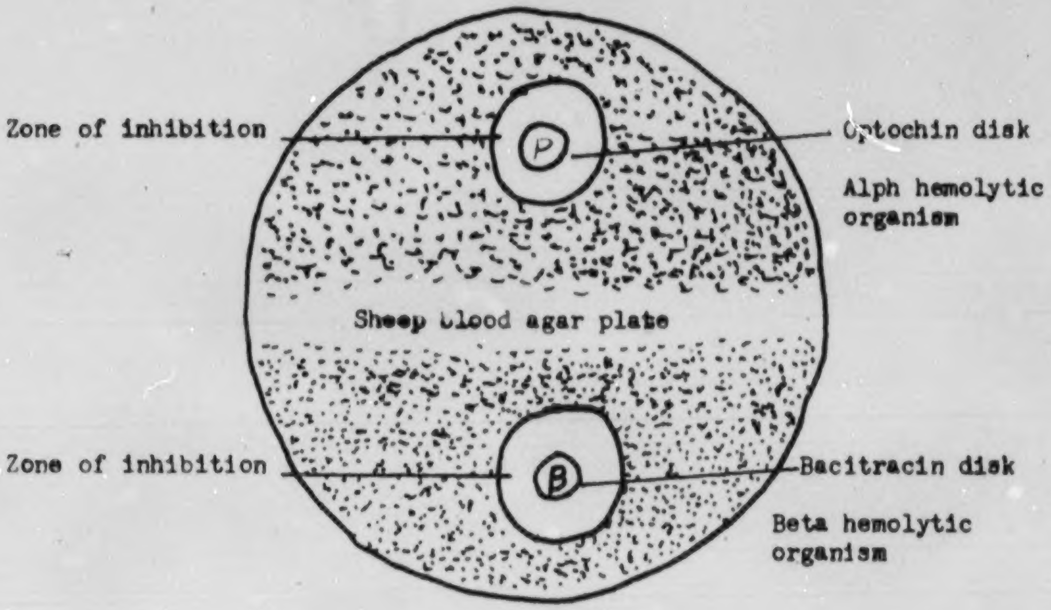
Magnetic Placards

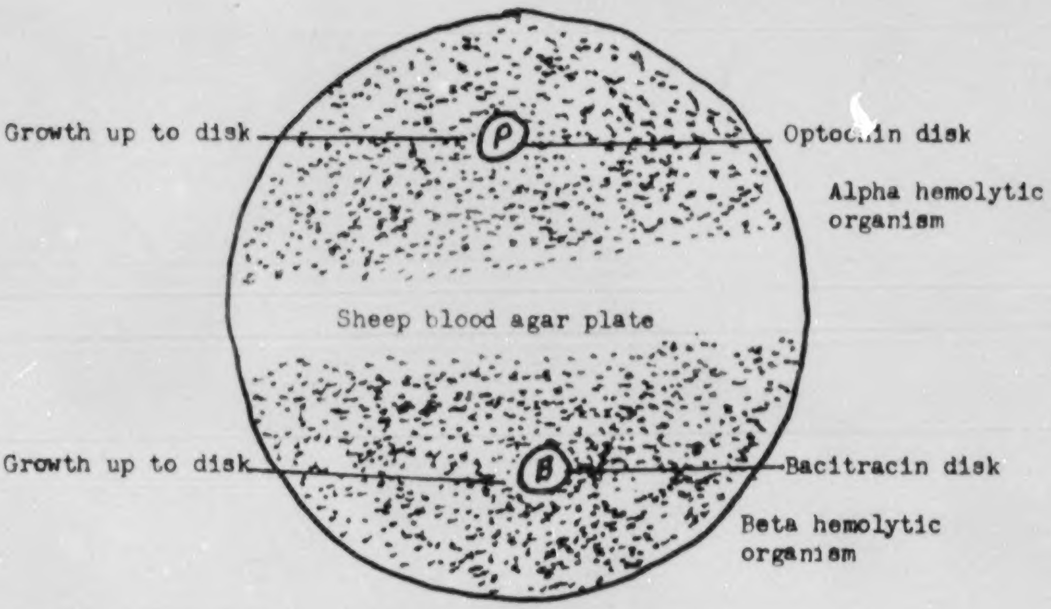
1. CATALASE
2. PURPOSE
3. REAGENT
4. INGREDIENTS
5. PERFORMANCE
6. INTERPRETATION
7. COAGULASE
8. SHEEP BLOOD AGAR
9. OXIDASE
10. BILE ESCULIN AZIDE
11. OPTOCHIN AND BACITRACIN

NOTE: These magnetic placards are used throughout the lecture to introduce topics.

ANNEX F
INSTRUCTIONAL AIDS

Chalkboard Diagram Optochin and Bacitracin

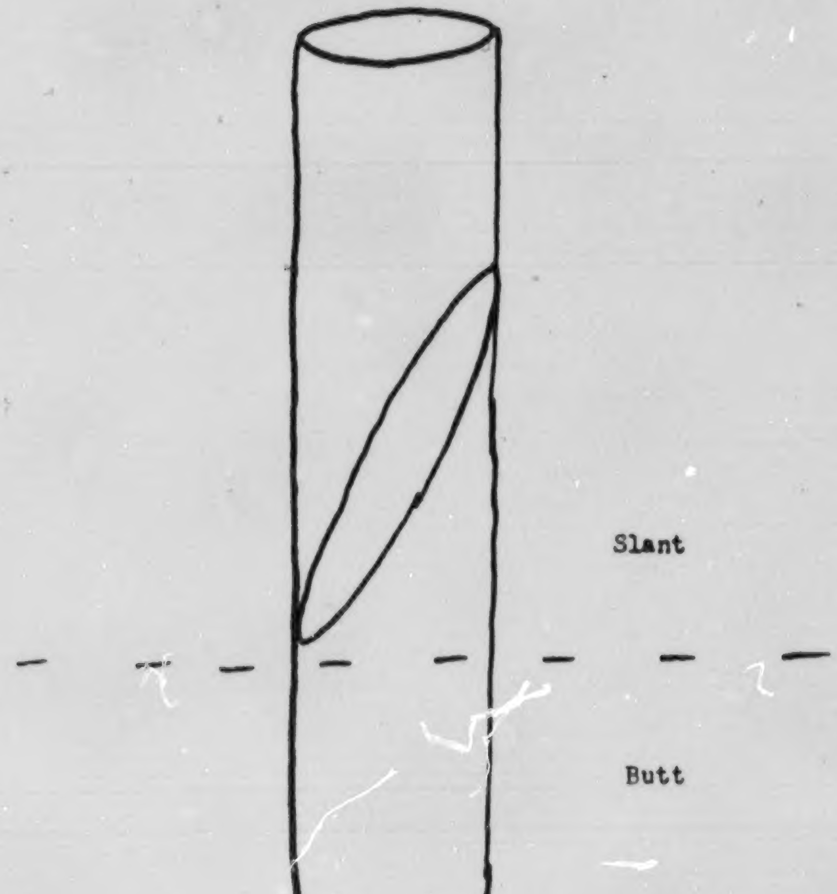

Zone of inhibition
Optochin disk
Alph hemolytic organism
P
Sheep blood agar plate
Zone of inhibition
B
Bacitracin disk
Beta hemolytic organism


Growth up to disk
P
Optochin disk
Alpha hemolytic organism
Sheep blood agar plate
Growth up to disk
B
Bacitracin disk
Beta hemolytic organism

Note; This diagram is used in part II E 4 f.

ANNEX G
INSTRUCTIONAL AIDS

Chalkboard Diagram Inoculation of BEA



NOTE: Draw diagram, explain slant and butt, and demonstrate inoculation procedure using a pointer as simulated inoculating needle. This diagram is used in Part II D 4.

SP 5 Calvin L. Mathews

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY LP 08-280-411
LABORATORY SCIENCES DIVISION 038
MICROBIOLOGY BRANCH

Biochemical Tests Practical Exercise - 4 hours

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Procedures, Basic

PLACE: Classroom

REFERENCES: All appropriate previous references.

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: See Annex A. M 08-280-407-1, prior to class.

PERSONNEL: Nine insturctors, MOS 92B3 H

INSTRUCTIONAL AIDS: Chalk, chalkboard, and eraser; see Annex A & B for equipment and materials.

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Practical exercise

I. INTRODUCTION: (5 min)

A. Opening Statement: During this exercise you will be performing biochemical tests that are used to identify bacteria. This exercise is designed to give you practice in performing procedures routinely used for identification of bacteria in the laboratory.

B. Objectives: Given bacterial cultures and necessary materials, perform, interpret, and report results of specified biochemical tests, as taught in class.

C. Class Procedure and Lesson Tie-In

1. Application of information received during previous lectures and demonstrations.

2. Practical exercise performing various biochemical tests will be conducted in four hours on two consecutive days. Specific

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*This LP supersedes LP 08-280-411, 016

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tasks assigned by the instructor during each phase will lead to completion of the exercise by the final hour.

3. Performed in conjunction with LP 08-280-407 and 08-280-409.

4. Biochemical tests are a crucial objective that will be tested later in the course.

II. APPLICATION (165 Min)

A. Instructors' Activities

1. Class divided into two-person teams
2. Equipment and materials issued (see Annex A)
3. Cultures issued (cultures from LP 08-280-407 will be used in this LP)
4. Daily instructions given
5. Previous day's activities reviewed
6. Questions answered
7. Corrections made
8. Laboratory safety maintained
9. Exercise classroom control

B. Students' Activities

1. Aseptic technique maintained
2. Biochemical tests performed
3. Culture materials discarded
4. Clean up performed as directed
5. Equipment stored

C. Organization of Instructional Hours

1. First Day

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a. Biochemical tests performed, inoculated, reported (see M 08-280-407-1).

b. Clean up as directed.

2. Second Day

a. Biochemical test interpreted and recorded.

b. Clean up performed

c. Report sheet reviewed for discrepancies

d. Review entire series of PE's 407, 409, and 411.

III. REVIEW (10 min)

A. Questions From Students

B. Review of Main Points

1. Inoculation of biochemical tests

2. Interpretation of biochemical tests

C. Closing Statement: Biochemical tests are the tools that a laboratory technician uses to identify bacteria. Without the mastery of these tools, he would not be able to complete his assigned tasks.

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ANNEX A

Tools, Equipment, and Materials

A. Items needed first day

1. Burner and tubing (one per student)
2. Instrument kit (one per student)
3. Glass slides (one box per student)
4. Disinfectant
5. Paper towels
6. Candle jars
7. Incubator
8. Inoculated plates from LP 08-280-407
9. Blood agar plate (one per student)

10. Pretested plasma, 0.5 ml per tube (one per student)
11. Dropper bottle, 30% H_2O_2 (four per class)
12. Optochin disks (four vials per class)
13. Bacitracin disks (four vials per class)
14. BEA slants (one per student)
15. Alcohol vial for flaming forceps (four per class)
16. Wax marker

B. Items needed for second day

1. Buckets for disposal of plates (six per class)
2. Autoclave

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ANNEX B

Instructional Aids

Organisms on Students Specimen Plates

Specimen	Organism
A-1	Alpha Streptococcus species
A-2	Beta Streptococcus not Gp A
A-3	Gamma Streptococcus Gp D
A-4	Staphylococcus aureus
A-5	Enterobacter species
B-1	Streptococcus pneumoniae
B-2	Beta Streptococcus Gp A
B-3	Gamma Streptococcus Gp D
B-4	Staphylococcus epidermidis
B-5	Neisseria species

ANNEX C

Tests to be Performed

SPECIMEN	TEST
A-1	Optochin susceptibility and catalase
A-2	Bacitracin susceptibility and catalase
A-3	Bile esculin azide and catalase
A-4	Catalase and coagulase
A-5	Catalase and oxidase
B-1	Optochin susceptibility
B-2	Catalase and Bacitracin susceptibility
B-3	Bile esculin azide
B-4	Catalase and coagulase
B-5	Oxidase

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SSG Philip A. Salerno

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-412
LABORATORY SCIENCES DIVISION 048
MICROBIOLOGY BRANCH

Streptococcus Species - 1 hour

COURSES PRESENTED TO: 311-92B20, Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES:

Bailey, W. R. & Scott, E. G. Diagnostic Microbiology. 4th ed. St. Louis: C. V. Mosby Co., 1974, ch 17 & 18.

Lennette E. H. and others (ed) Manual of Clinical Microbiology. 2nd ed. Bethesda: American Society for Microbiology. 1974 ch 8 & 9

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: None

PERSONNEL: One instructor MOS 92B4H

INSTRUCTIONAL AIDS: Chalk, eraser, magnetic chalkboard, MP's (see Annex A)

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Lecture

I. INTRODUCTION (5 min)

A. Opening Statement: Members of the genus Streptococcus are frequently isolated from clinical specimens and some species are common pathogens. Becuase of this, it is essential that the clinical bacteriologist successfully cultivate and identify members of this genus.

B. Objective:

1. Given criteria used in the identification of various

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*This lesson plan supersedes LP 08-280-412, 046

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bacterial species, to include nutritional and environmental requirements, colony morphology, and biochemical test reactions; select criteria that are used in the identification of the Streptococcus species.

2. Given a list of specimen sources and pathogenic processes, select the source and/or process where the Streptococcus species may be a disease-causing agent.

C. Class Procedure and Lesson Tie-in

During the sebsequent practical exercises, students will cultivate and identify members of the genus Streptococcus, using principles discused during lecture.

II. EXPLANATION: (30 min)

A. Growth Requirements

1. Sheep blood agar - Streptococcus species are fastidious and will not grow on basal medium

QUESTION: What does the term fastidious mean?

ANSWER: The term fastidious means the organism requires special nutrients.

2. Increased CO_2

3. Various oxygen tensions - facultative

4. Incubate at 37°C for 18-24 hours

B. Identification Procedures

1. Gram morphology

a. Gram positive

b. Cocci

c. Chains

d. Pairs - can be in a lancet-shape

2. Catalase negative

3. Hemolytic patterns

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a. Alpha hemolysis

- (1) Possible organisms
    - (a) *Streptococcus pneumoniae*
    - (b) Alpha *Streptococcus* species not group D
    - (c) Group D alpha *Streptococcus* species
- (2) Tests performed on alpha hemolytic organisms
    - (a) Optochin susceptibility test
    - (b) Bile esculin azide test (BEA)
- (3) Identification based on test results
    - (a) Optochin susceptible, BEA negative--*Streptococcus pneumoniae*
    - (b) Optochin resistant, BEA negative--Alpha *Streptococcus* species not group D
    - (c) Optochin resistant, BEA positive--Group D alpha *Streptococcus* species

QUESTION: What color is a positive BEA?

ANSWER: The color of a positive BEA is black.

NOTE: Mention that a BEA should be inoculated at the same time a susceptibility test is performed

NOTE: The above information may be presented on the chalkboard in the following table fashion:

a. Alpha hemolysis	Tests and Results	
Possible organisms	Optochin	BEA
Streptococcus pneumoniae	Susceptible	Negative
Alpha *Streptococcus* species not group D	Resistant	Negative
Group D alpha *Streptococcus* species	Resistant	Positive

NOTE: The following organism information may also be presented in a simular fashion for ease of student understanding.

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b. Beta hemolysis

(1) Possible organisms

(a) Group A beta Steptococcus species

(b) Group D beta Streptococcus species

(c) Beta Streptococcus species not group A or D

(2) Tests performed on beta Hemolytic organisms

(a) Bacitracin susceptibility test

(b) Bile esculin azide agar (BEA)

(3) Identification based on test results

(a) Bacitracin susceptible, BEA negative--Group A beta Streptococcus species

(b) Bacitracin resistant, BEA positive--Group D beta Streptococcus species

(c) Bacitracin resistant, BEA negative--beta Streptococcus species not group A or D

c. Gamma hemolysis

(1) Possible organisms

(a) Group D gamma Streptococcus species

(b) Gamma Streptococcus species not group D

(2) Test performed on gamma hemolytic organisms

(a) Bile esculin azide agar (BEA)

(3) Identification based on test results

(a) BEA positive--Group D gamma Streptococcus species

(b) BEA negative--Gamma Streptococcus species not group D

C. Clinical Importance and Specimen Source

1. Streptococcus pneumoniae
    a. Lobar pneumonia--sputum
    b. Meningitis--Spinal fluid
2. Group A beta Streptococcus species
    a. "Strep throat"--throat
    b. Scarlet fever
    c. Glomerulonephritis
    d. Rheumatic fever
3. Group D Streptococcus species
    a. Urinary tract infection--urine
    b. Respiratory tract infection--sputum
    c. Endocarditis--blood

D. Questions From Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Growth requirements
2. Identification procedures
3. Clinical importance and Specimen Source

B. Closing Statement: By employing the characteristics we have discussed, members of the genus Streptococcus can be cultivated and idetified with relative ease.

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LP 08-280-412
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ANNEX A
INSTRUCTIONAL AIDS

Magnetic Placards

MP 08-280-412-1	IDENTIFICATION OF STREPTOCOCCUS SP
MP 08-280-412-2	NUTRITIONAL REQUIREMENTS
MP 08-280-412-3	POSSIBLE ORGANISMS
MP 08-280-412-4	GRAM MORPHOLOGY
MP 08-280-412-5	ALPHA HEMOLYSIS
MP 08-280-412-6	BETA HEMOLYSIS
MP 08-280-412-7	GAMMA HEMOLYSIS
MP 08-280-412-8	CLINICAL SIGNIFICANCE

35mm Slides

S 08-280-412-1	alpha hemolytic Streptococcus sp. colony on blood agar
S 08-280-412-2	beta hemolytic Streptococcus sp. colony on blood agar
S 08-280-412-3	gamma hemolytic Streptococcus sp. colony on blood agar
S 08-280-412-4	Susceptible bacitracin and optochin tests
S 08-280-412-5	Positive bile esculin azide agar test

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *IM 08-280-412
LABORATORY SCIENCES DIVISION 048
MICROBIOLOGY BRANCH

Streptococcus Species

Members of the genus Streptococcus are frequently isolated from clinical specimens and some species are common pathogens. Because of this, it is essential that the clinical bacteriologist successfully cultivate and identify members of this genus.

The first concern in cultivating any organism from a clinical specimen is the nutritional requirement of the organism that is expected to be isolated. Members of the genus Streptococcus are fastidious. Sheep blood is usually used when there is a possibility that a Streptococcus species might be isolated. Increased CO_2 is also a nutritional requirement. Members of this genus are either aerobic, microaerophilic, or facultative. The streptococci should be incubated at 37°C for 18 to 24 hours.

The colony morphology of the Streptococcus species after 18 to 24 hours at 37°C on sheep blood agar is a factor that is essential to identification. All three hemolytic patterns (alpha, beta, and gamma) may be produced by various species. The colonies are typically small and translucent to slightly opaque.

The gram morphology of Streptococcus is the next step in identification. The Streptococci are gram positive cocci typically occurring in chains or pairs.

The identification of the genus depends on the gram morphology and the catalase reaction. Streptococci are catalase negative.

In the differentiation of the Streptococcus species, hemolysis is the primary differentiation characteristic. This will divide the genus into alpha Streptococcus species, beta Streptococcus species, and gamma Streptococcus species.

Biochemical testing is the secondary method of differentiation. Optochin susceptibility is performed on alpha hemolytic species only. If the organism is susceptible to optochin it is reported as Streptococcus pneumoniae. If the organism is resistant to optochin it is reported as alpha Streptococus species. Bacitracin susceptibility is performed on beta hemolytic species only. If the organism is susceptible to bacitracin it is reported as Gp A, beta Streptococcus species. If the organism is resistant to bacitracin it is reported as beta Streptococcus species, not Gp A.

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*This instructor's manuscript supersedes IM 08-280-412, 046

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Bile Esculin Azide Agar testing is performed on all hemolytic groups of *Streptococcus*. If the organism is BEA positive, report it as Gp D *Streptococcus* species. If the organism is BEA negative, it is reported as not Gp D *Streptococcus* species. The hemolytic pattern of the organism is included in the report of the test for Gp D.

Serological testing is the tertiary differentiation procedure for the *Streptococci*. This is the most definitive method of differentiation.

The genus *Streptococcus* includes several pathogens. Gp A *Streptococcus* sp cause "Strep throat," scarlet fever, glomerulonephritis, and sepsis. Gp D *Streptococcus* species cause urinary tract infections, endocarditis, sepsis, and respiratory tract infections. *Streptococcus pneumoniae* causes lobar pneumonia, meningitis, and sepsis. Alpha *Streptococcus* species can cause subacute bacterial endocarditis.

By employing the characteristics we have discussed, members of the genus *Streptococcus* can be cultivated and identified with relative ease.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCE DIVISION
MICROBIOLOGY BRANCH

LP 08-280-413
038

The Throat Specimen (1 hour)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Procedures Course (Basic)

PLACE: Classroom

REFERENCES:

Bailey, W. Robert, and Scott, Evelyn G., Diagnostic Microbiology. 3d ed. St. Louis: C. V. Mosby Company, 1974, Chapter 8.
Lennett, E. H. and others (ed.). Manual of Clinical Microbiology. 2d ed. Washington: American Society for Microbiology, 1974, Chap 6.

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: Not applicable

PERSONNEL: One instructor (MOS 92B3H)

INSTRUCTIONAL AIDS: Chalk, magnetic chalkboard, and eraser.

TROOP REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

TRANSPORTATION REQUIREMENTS: None

I. INTRODUCTION (5 min)

A. Opening Statement: Throat specimens are routinely submitted for the identification of the organisms causing streptococcal sore throat. Improper identification and/or treatment can result in scarlet fever, rheumatic fever, or glomerulonephritis. Throat specimens are also submitted to check for bacterial pneumonia, diptheria, whooping cough and meningitis. Screening for Group A beta hemolytic Streptococcus is of primary concern when working with throat specimens, and every laboratory technician must develop the necessary skills to isolate and identify the above organism.

This lesson plan new to PIO

B. Objectives:

1. Given a situation involving the collection and processing of a throat specimen, select the correct steps used in the proper collection and processing of a throat specimen.

2. Given a list of bacteria, select the bacteria most commonly found in a throat specimen.

3. Given a list of reporting terminologies and various types of bacterial flora, select the correct reporting terminology for the various types of bacteria.

C. Class Procedure and Lesson Tie-in:

A practical exercise follows this lecture wherein students will screen specimens for Group A beta hemolytic Streptococci utilizing the identification procedures discussed in this lecture.

II. EXPLANATION (35 min)

A. Description of Specimen

1. Collected on a sterile cotton swab

NOTE: Commercial preparations containing a swab and an ampule of modified Stuart transport medium is preferred over a dry swab.

2. Specimen collected from lower area at back of oral cavity or tonsils

B. Collection of Specimen

1. Collected by physician, nurse, or laboratory technician.

2. Depress tongue with sterile depressor blade.

3. Vigorously swab over tonsils and back of throat.

4. Avoid touching tongue or lips.

5. Replace swab in tube, crush ampule if using diposable unit.

6. Send to laboratory immediately for processing.

C. Processing

1. Inoculate swab to sheep blood agar

2. Streak inoculated agar for isolation

3. Stab agar 5 to 10 times to detect subsurface hemolysis
(due to production of streptolysin O)

4. Incubate 18 to 24 hours at 37°C.

5. Use increased CO_2 conditions (candle jar or CO_2 incubator)

QUESTION: What is the primary isolation medium for a routine throat specimen?

ANSWER: Sheep blood agar.

D. Identification of Beta Hemolytic Organisms

1. Screen the sheep blood agar plates for beta hemolytic colonies

2. Do a catalase test on any beta hemolytic colony

3. Do a bacitracin test on any catalase negative colony

4. Reporting - presumptive identification

   a. No beta hemolytic colonies on original plate-
   report "no Beta Streptococci isolated"

   b. Beta hemolytic colonies that are catalase positive-
   report "no Beta Streptococci isolated"

   c. Beta hemolytic colonies, that are bacitracin resistant-
   report "Beta Streptococci not Group A isolated"

   d. Beta hemolytic colonies that are bacitracin susceptible-
   report "presumptive Group A Beta Streptococci isolated"

5. Definitive identification of Beta Streptococci species

   a. Serological grouping

   b. Fluorescent antibody procedure

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E. Normal Throat Cultures

1. Predominance of alpha hemolytic Streptococci and Neisseria species

2. Other organisms seen

   a. Staphylococcus species

   b. Haemophilus species

   c. Streptococcus pneumoniae

   d. Gamma Streptococcus species

   e. Diphtheroid and coliform bacilli

F. Miscellaneous Pathogens

1. Corynebacterium diphtheriae - etiological agent of diphtheria

2. Bordetella pertussis - etiological agent of whooping cough

3. Neisseria meningitidis - etiological agent of meningitis

QUESTION: The genus involved in the disease diphtheria is?

ANSWER: Corynebacterium.

G. Questions from Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Description

2. Collection

3. Processing

4. Identification of beta hemolytic organisms

5. Normal throat cultures

6. Miscellaneous pathogens

B. Closing Statement: Interpretation of throat cultures is at times difficult because of the normal flora, but every laboratory recognizes the significance of Group A beta hemolytic Streptococci. A new laboratory technician will become familiar with this screening procedure in a few days, especially during the winter months when throat specimens are routinely submitted for colds and sore throats.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-414
LABORATORY SCIENCE DIVISION 038
MICROBIOLOGY BRANCH

Staphylococcus Species - 1 hour

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Procedures, Basic

PLACE: Classroom

REFERENCES:

Bailey, W. Robert, and Scott, Elvyn G. Diagnostic Microbiology. 3rd ed. St. Louis: C.V. Mosby Company, chap 16, 1974.
Lennette, E.H. and others, Manual of Clinical Microbiology. 2nd ed. Washington: American Society for Microbiology, chap 7, 1974.

STUDY ASSIGNMENT: None.

STUDENT UNIFORM AND EQUIPMENT: Not applicable.

PERSONNEL: One instructor, MOS 92B3H

INSTRUCTIONAL AIDS: Chalk, magnetic chalkboard, eraser; projector, screen; S 08-280-414-1 thru -6 (see Annex B); magnetic placards (see Annex A)

TROOP REQUIREMENTS: None.

TRANSPORTATION REQUIREMENTS: None.

METHOD OF INSTRUCTION: Lecture.

I. INTRODUCTION (5 min)

A. Opening Statement: Members of the genus Staphylococcus are capable of invading and causing disease in any part of the body. Members of this genus commonly are isolated from boils, wounds, blood cultures, and are a leading cause of food poisoning in the U.S. During this hour you will learn the characteristics and identification procedures of the genus Staphylococcus and the species within this genus.

B. Objectives

1. Given criteria used in the identification of the Staphylococcus species, to include nutritional and environmental requirements, colony morphology, and biochemical test reactions, select criteria that are used in the differentiation of the Staphylococcus species.

2. Given a list of specimen sources and pathogenic processes,

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*This lesson plan supersedes LP 08-280-414, 046

select the source and/or process where the Staphylococcus species may be a disease-causing agent.

C. Class Procedure and Lesson Tie-in: Students will work with and identify Staphylococcus species in subsequent practical exercises and examinations, using characteristics discussed in this lecture.

II. EXPLANATION (35 min)

A. Nutritional and Environmental Requirements

1. Non-fastidious growth on basal media

NOTE: Though not required, stapnylococci grow well on enriched media.

2. Temperature - optimum 35 to 37°C

3. Aerobic to facultative anaerobic

4. Tolerate high salt (NaCl) concentration

a. Up to 10%

b. Termed haloduric

NOTE: In some literature the term halophilic is used though this is not truly descriptive of Staphylococcus species. They are not "salt loving" but "salt enduring" bacteria.

QUESTION: What is the optimum temperature for the growth of Staphylococcus species?

ANSWER: 35 to 37°C

5. May tend to overgrow fastidious bacteria.

B. Colony Morphology on Sheep Blood Agar

NOTE: Staphylococcus species are usually isolated on SBA, inoculated and incubated 18 to 24 hours at 37°C.

1. Size - medium to large (2-4 mm diameter)

2. Opaque

3. Pigmentation

a. White

- b. Yellow
- c. Gold

4. Hemolysis
    - a. Gamma
    - b. Beta

C. Gram Morphology

1. Gram positive
2. Cocci
3. Arrangement
    - a. Irregular grape-like clusters
    - b. Single cells, pairs, short chains

D. Genus Characteristics

1. Enzyme catalase produced

QUESTION: What occurs when 30 percent hydrogen peroxide is added to *Staphylococcus* species which are placed on a clean glass slide?

ANSWER: There will be immediate and vigorous bubbling.

2. Growth on media containing up to 10% NaCl.
3. Two species
    - a. *Staphylococcus aureus*
    - b. *Staphylococcus epidermidis*

E. Species Characteristics

1. *Staphylococcus aureus*
    - a. Ferments the carbohydrate mannitol to produce an acid.

b. Enzyme coagulase produced

(1) Plasma clotted

(2) Means by which organism is identified as Staphylococcus aureus

c. Beta hemolytic (usually)

NOTE: Stress that Staphylococcus aureus is identified by the coagulase test result, since mannitol fermentation and colony morphology may vary.

2. Staphylococcus epidermidis

a. No fermentation of mannitol

b. Enzyme coagulase not produced

(1) Plasma not clotted

(2) Means by which organism is identified as Staphylococcus epidermidis

c. Gamma hemolytic (usually)

NOTE: Hemolysis and pigmentation are not criteria to be used for differentiation of Staphylococcus aureus from Staphylococcus epidermidis.

QUESTION: How do we distinguish Staphylococcus from Streptococcus species?

ANSWER: The catalase test result combined with other genus characteristics are used.

F. Clinical importance

1. Staphylococcus aureus

a. Production of variety of toxins

b. Pathogenic

c. Diseases or infections
    (1) Boils
    (2) Wound infections
    (3) Acne
    (4) Pneumoniae
    (5) Septicemia
    (6) Meningitis
    (7) Food poisoning
        (a) Enterotoxin-thermostable
        (b) Most common cause in US
    (8) Urinary tract infections

d. Specimens
    (1) Swabs
        (a) Nose
        (b) Throat
        (c) Abscess
        (d) Wound
    (2) Pus
    (3) Sputum
    (4) Blood
    (5) Spinal fluid
    (6) Food
    (7) Urine

2. Staphylococcus epidermidis

a. No toxins

b. Potential pathogen

NOTE: S. epidermidis is present on skin and may act as an opportunistic pathogen.

c. Diseases or infections

(1) Stitch abscesses

(2) Skin infections

(3) Sinusitis

(4) Bacteremia

(5) Urinary tract infections

d. Specimens

(1) Swabs

(2) Blood

(3) Urine

(4) Other specimens

(a) Contaminant

1 Collection

2 Processing

(b) Normal flora on the skin

(c) Significance determined by physician

NOTE: Show slides 08-280-414-1 thru -5.

G. Questions from Students

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III. SUMMARY (5 min)

A. Review of Main Points

1. Nutrition and environmental requirements

2. Colony morphology

3. Gram morphology

4. Genus characteristics

5. Species characteristics

6. Clinical importance

B. Closing Statement: Because of the disease-producing capability of some staphylococci and their variation in response to antibiotics it is vital to isolate and identify members of this genus efficiently and accurately.

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LP 08-280-414
038

ANNEX A
INSTRUCTIONAL AIDS

Magnetic Placards

1. Identification of Staphylococcus species
2. Nutritional and Environmental Requirements
3. Colony Morphology
4. Gram Morphology
5. Genus Characteristics
6. Staphylococcus aureus
7. Staphylococcus epidermidis
8. Species Characteristics
9. Clinical Importance

NOTE: These will be used as needed in Part II.

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ANNEX B
INSTRUCTIONAL AIDS

35 mm Slides

1. Gram stain of *Staphylococcus* species.
2. White colony of *Staphylococcus* species on SBA.
3. Yellow colony of *Staphylococcus* species on SBA.
4. Positive catalase test.
5. A positive and a negative coagulase test.

NOTE: These will be shown in Part II.

SSG Bertie Bake

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-415
LABORATORY SCIENCES DIVISION 048
MICROBIOLOGY BRANCH

Identification of Neisseria Species - 1 hour

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES:

Bailey, W. Robert, and Scott, Elvyn G. Diagnostic Microbiology. 4th ed. St. Louis: C.V. Mosby Company, 1974, chap 19.
Lennette, E. H. et al (eds.) Manual of Clinical Microbiology, 2d ed. Washington: American Society for Microbiology. 1974, chap 10 & 11.

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: None

PERSONNEL: One primary instructor (MOS 92B30)

INSTRUCTIONAL AIDS: Chalk, chalkboard, and eraser; 35 mm Carousel projector; screen; S 08-280-415-1 thru -5; and magnetic placards, MP 08-280-415-1 thru -15 (see Annex A).

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Lecture

I. INTRODUCTION (5 min)

A. Opening Statement: Two species of the genus Neisseria are the causative agents of diseases which have considerable impact upon our society and the army today. Gonorrhea is the most prevalent of the venereal diseases and meningococcal meningitis has been a serious problem, especially at military basic training centers.

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*This lesson plan supersedes LP 08-280-415, 016.

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B. Objectives:

1. Given criteria used in the identification of various bacterial species, to include nutritional and enviromental requirements, growth characteristics, and biochemical test reactions, select criteria that are used in the identification of the Neisseria species.

2. Given a list of specimen sources, and pathogenic processes, select the source and/or the process where the Neisseria species may be a disease-causing agent.

C. Class Procedure and Lesson Tie-in

1. Practical exercises with known Neissera species to follow for reinforcement of lecture material.

2. Unknown practical exercises conducted during ensuing weeks to improve proficiency in isolating and identifying Neisseria species.

II. EXPLANATION (35 min)

A. Genus Characteristics

1. Seven species in the genus

a. Two pathogens

(1) Neisseria gonorrhoeae

(2) Neisseria meningitidis

b. Five nonpathogens--normal flora in upper respiratory tract

2. Gram negative diplococci

a. Coffee bean-shaped

b. Adjacent sides flattened

3. Catalase positive

4. Oxidase positive

5. Acid produced from different carbohydrates

B. Colony Morphology

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B. C

1. Nonpathogens on sheep blood agar (18-24 hours)
    - a. Nonhemolytic--gamma
    - b. Dry, crusty--smooth
    - c. Medium size
    - d. Gray-white to yellow
2. Pathogens on appropriate medium
    - a. Smooth to mucoid
    - b. Small to medium
    - c. Cream to gray color
    - d. Glistening

C. Growth requirements of Pathogens and Nonpathogens

1. Nonpathogenic species
    - a. Grow on basal medium at 22 C to 37 C
    - b. Grow on SBA
2. Pathogenic species
    - a. Fastidious--need special enrichments
    - b. No growth on basal medium
    - c. No growth at 22 C
    - d. Optimal temperature 35 C (range 35 -37 C)
    - e. Increased CO_2 (5 to 10%) enhances initial isolation
    - f. Require moisture--moist gauze in candle jar
    - g. Incubation 18 to 48 hours

__NOTE:__ N. meningitidis grows readily on SBA. __N. gonorrhoeae__ has limited growth on SBA.

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D. Enriched Media for Isolation of Pathogens

1. Chocolate agar--medium of choice

a. Blood or hemoglobin (2%)

b. Supplement A or B (1%)

2. Thayer-Martin agar

a. GC agar base

b. Hemoglobin

c. V-C-N inhibitor

d. IsoVitale X enrichment

NOTE: V-C-N inhibits the growth of most organisms including all Neissera species except N. lactamica, N. gonorrhoeae, and N. meningitidis

E. Laboratory Identification

1. Presumptive identification

a. Oxidase positive

b. Gram stain reaction

c. Source of specimen

(1) Cerebral spinal fluid--N. meningitidis

(2) Urethral exudate--N. gonorrhoeae

d. Initial Gram stain on urethral exudate--if positive report "intracellular and extracellular Gram negative diplococci seen".

2. Definitive identification

a. Carbohydrate fermentation patterns

(1) Cystine trypticase agar (CTA)

(a) Glucose 1.0 percent

(b) Maltose 1.0 percent

(2) Arthritis

(3) Endocarditis

(4) Opthalmia of the newborn

d. Specimens

(1) Urethral and vaginal exudates

(2) Synovial fluid

(3) Blood

(4) Eye exudate

G. Questions From Students

III. SUMMARY

A. Review of Main Points

1. Genus characteristics

2. Colony morphology

3. Growth requirements

4. Enriched media for isolation of pathogens

5. Laboratory identification

6. Clinical importance

B. Closing Statement: Because of the disease-causing capability of certain Neisseria species, it is important for the medical laboratory specialist to be able to isolate and identify these organisms with speed and accuracy.

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- (c) Sucrose 1.0 percent
- (d) Lactose 1.0 percent
- (e) Phenol red indicator

(2) Inoculate with pure culture of Neisseria

(3) Do not use increased CO_2

(4) Check for different fermentation patterns

b. Serological grouping--for most species

F. Clinical Importance

1. Nonpathogens

   a. Normal flora of throat and nasopharynx

   b. Not usually clinically significant

2. Neisseria meningitidis

   a. Inhabits nasopharynx

   b. Diseases caused

      (1) Meningitis

      (2) Septicemia

   c. Specimens

      (1) Throat swab

      (2) Spinal fluid

      (3) Blood

3. Neisseria gonorrhoeae

   a. Most prevalent venereal disease

   b. Sexual transmission

   c. Diseases

      (1) Venereal disease

ANNEX A
INSTRUCTIONAL AIDS

Slides

S 08-280-415-1	A color slide of typical Neisseria colonies on sheep blood agar.
S 08-280-415-2	A color slide of typical Neisseria colonies on sheep blood agar (close up).
S 08-280-415-3	A color slide of typical Neisseria colonies on sheep blood agar (start of oxidase).
S 08-280-415-4	A color slide of a positive oxidase test performed on colonies of Neisseria growing on sheep blood agar.
S 08-280-415-5	A color slide of a positive GC smear.

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ANNEX B
INSTRUCTIONAL AIDS

Magnetic Placards

1. IDENTIFICATION OF NEISSERIA SPECIES
2. NUTRITION AND CULTIVATION
3. NONPATHOGENS
4. PATHOGENS
5. COLONY MORPHOLOGY
6. GRAM MORPHOLOGY
7. GENUS CHARACTERISTICS
8. NEISSERIA GONORRHOEAE
9. NEISSERIA MENINGITIDIS
10. SPECIES CHARACTERISTICS
11. TENTATIVE IDENTIFICATION
12. DEFINITIVE IDENTIFICATION
13. CLINICAL SIGNIFICANCE

NOTE: These placards are used throughout the lesson plan at major headings.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-416
LABORATORY SCIENCE DIVISION 038
MICROBIOLOGY BRANCH

Throat Specimen Practical Exercise - 4 hours

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Procedures, Basic

PLACE: Classroom

REFERENCES: All appropriate references

STUDY ASSIGNMENT: Before class read M 08-280-416-1

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: See Annexes A and B.

PERSONNEL: Eight instructors, MOS 92B3H.

INSTRUCTIONAL AIDS: Chalk, chalkboard, and eraser.

TROOP REQUIREMENTS: None.

TRANSPORTATION REQUIREMENTS: None.

METHOD OF INSTRUCTION: Practical exercise.

I. INTRODUCTION (5 min)

A. Opening Statement: During this exercise, the remainder of this course, and after your assignment to clinical laboratories, you will be required to screen throat specimen plates for the presence of Beta hemolytic Streptococcus. This exercise is designed to give you practice in performing procedures routinely used for the isolation and identification of Beta hemolytic Streptococcus.

B. Objective: Given bacterial specimens and all necessary materials, inoculate and streak media for isolation; interpret and report colony morphology; perform and report all tests necessary to identify Beta hemolytic Streptococcus, as demonstrated in class.

C. Class Procedure and Lesson Tie-in

1. Application of information received during previous lectures and demonstrations.

2. This practical exercise with known species of Staphylococcus, Streptococcus, and Neisseria genera will be conducted in

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*This lesson plan supersedes LP 08-280-416, 016

four hours on three consecutive days; one hour the first day, two hours the second day, and one hour the third day. Specific tasks assigned by instructor during each pahse will lead to completion of the exercise by the final hour.

II. APPLICATION (145 min)

A. Instructors' Activities

1. Class divided into teams of two students each.
2. Equipment and materials issued (see Annexes A and B).
3. Cultures prepared and issued (see Annex C, day prior to PE)
4. Daily instructions given in step-by-step fashion.
5. Previous day's activities reviewed.
6. Questions answered.
7. Corrections made.
8. Laboratory safety maintained.
9. Exercise classroom control.

B. Student's Activities

1. Aseptic technique maintained.
2. Appropriate media inoculated and streaked for isolation (see M 08-280-416-1).
3. Colony morphology observed and recorded (see M 08-280-416-1).
4. Biochemical tests performed.
5. Culture materials discarded.
6. Clean up as directed.
7. Equipment stored properly.

C. Organization of Instructional Hours

1. First Day (35 min) (see Annexes A, B, and C)

a. Selection of cultures by students

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NOTE: Each group of two students receives eight specimens divided into two groups of four each, labeled A and B. Each member of every group will receive four specimens. All different hemolytic patterns will be represented.

b. Cultures inoculated and streaked (see M 08-280-416-1)

c. Cultures incubated

d. Specimens properly discarded.

e. Clean-up performed

2. Second Day (80 min) (see Annexes A and B)

a. Colony morphology observed and recorded (see all organisms)

b. Catalase test performed an all beta hemolytic organisms

c. Perform all appropriate biochemical tests (see M 08-280-416-1)

d. Freshly inoculated media incubated

e. Original plates refrigerated

f. Clean-up performed

3. Third Day (30 min) (see Annexes A and B)

a. Biochemical tests interpreted and results recorded

NOTE: Refer students to appropriate mimeos for assistance in test interpretation.

b. Review report sheet for discrepancies

c. All culture materials properly discarded

d. Clean-up performed

III. REVIEW (10 min)

A. Questions from Students

B. Review of Main Points and Work Sheet

1. Colony morphology and hemolysis

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2. Catalase as an aid in selecting biochemical tests

3. Biochemical reactions and recording

4. Proper identification procedures

C. Closing Statement: Background knowledge of the theories and principles of clinical bacteriology, though very important, is insufficient. You must develop the ability to apply that knowledge in an organized and orderly manner so that you can satisfactorily perform your duties as a clinical bacteriology technician.

ANNEX A

Tools, Equipment, and Materials

1.	Autoclave, laboratory	1 per class
2.	Refrigerator	1 per class
3.	Incubator, CO_2	1 per class
4.	Microscope, binocular complete	1 per student
5.	Loop, inoculating	1 per student
6.	Needle, inoculating	1 per student
7.	Forceps, flat tips	1 per student
8.	Marker, wax	1 per student
9.	Glass slides	1 box per student
10.	Disinfectant, germicidal	2 gallons per class
11.	Detergent, surgical	1 gallon per class
12.	Racks, test tube, 90-hole	1 per student
13.	Burner, gas, touchmatic	1 per student
14.	Bucket, metal, decontamination	4 per class
15.	Towels, paper	7 pks per class
16.	Immersion oil, microscope	1 per student
17.	Rack, Gram staining, complete	7 per class
18.	Tape, masking	3 rolls per class
19.	Canister, metal, plate	1 per student
20.	Candle jar, complete	10 per class
21.	Dishwasher	1 per class
22.	Swabs, sterile	4 per student
23.	Racks, test tube, 40 hole	20 per class

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ANNEX B

Media and Reagents

1. Sheep Blood Agar (SBA)	6 per student
2. Bacitracin discs	4 vials per class
3. Alcohol bottles (for flaming forceps)	4 per class
4. 30% Hydrogen Peroxide (H_2O_2)	4 bottles per class
5. Gram stain reagents (set)	1 per sink
6. Todd-Hewitt broth (100 ml/bottle)	5 bottles

ANNEX C

Procedure for Preparation of Practical Exercise

A. Materials

1. Six bottles of Todd-Hewitt broth (100 ml/bottle)
2. Sheep Blood Agar cultures
    a. *Staphylococcus aureus*
    b. Alpha hemolytic *Streptococcus* species not group D
    c. Group A beta hemolytic *Streptococcus* species
    d. Beta hemolytic *Streptococcus* species not group A or D
    e. Gamma *Streptococcus* species group D
3. Forty-hole racks (one per group of two students)
4. Swab tubes (sterile) four per student
5. Masking tape (1 roll)
6. Burner with tubing
7. Felt tip marker
8. Sterile swabs and inoculating loop
9. Disinfectant

B. Preparation Procedure

1. Day prior to practical exercise
    a. Inoculate Todd-Hewitt broths with organisms from appropriate culture plates, using a sterile loop. Insure that all bottles are labeled appropriately. Inoculate two bottles with the alpha *Streptococcus* species, not group D.
    b. Incubate all inoculated media

2.. Day of PE, Prepare bacterial mixtures; dip swab tubes into appropriate mixtures and distribute in accordance with Protocol #1 (one rack of specimens per group of two).

3. Mixtures

#1: Alpha Streptococcus sp and Beta Streptococcus sp group A.

#2: Alpha Streptococcus sp and Beta Streptococcus sp not gp A.

#3: Alpha Streptococcus sp and Gamma Streptococcus sp, group D.

#4: Alpha Streptococcus sp and Staphylococcus aureus.

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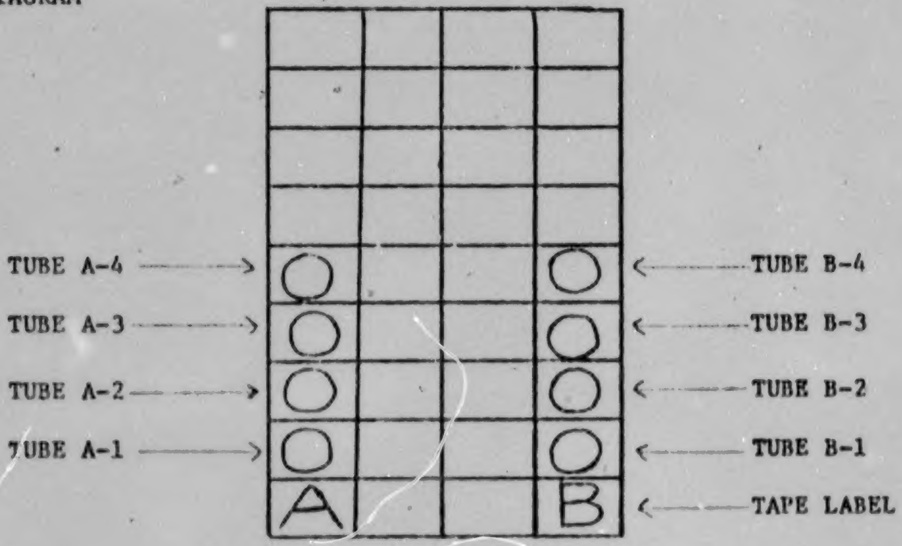
PROTOCOL 1

	TUBE	MIXTURE #
GROUP A	A-1	1
	A-2	2
	A-3	3
	A-4	4
GROUP B	B-1	3
	B-2	1
	B-3	4
	B-4	2

DIAGRAM


TUBE A-4
TUBE A-3
TUBE A-2
TUBE A-1
A
B
TUBE B-4
TUBE B-3
TUBE B-2
TUBE B-1
TAPE LABEL

NOTE: One rack arranged in this manner is needed for every two students. Care should be taken to arrange organisms exactly as indicated so that they will match the students instruction sheet (M 08-280-416-1).

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LABORATORY SCIENCE DIVISION
MICROBIOLOGY BRANCH

*M 08-280-416-A
038

Procedure for Throat Specimen - Practical Exercise

First Day

1. The class will be divided into groups of two students each. Each group will be issued eight cultures in one 40-hole rack as indicated in figure 1, below. Each student in the group should choose either A or B.

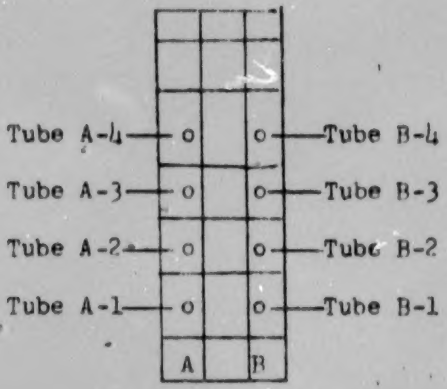

Figure 1

2. Each student will obtain four sheep blood agar plates(SBA) and label one for each culture on the back of the plates using a wax marker. The label should include students name, date, and culture number (e.g., A-1, A-2, etc).

3. Using aseptic technique, inoculate the labeled media by rolling the swab on a small portion of the surface of the appropriately labeled media. Streak all SBA plates for isolation using the four quadrant method.

4. Stack the inverted SBA plates in a metal canister and incubate the plates in a CO_2 incubator or candle jar as directed by an instructor.

5. Clean-up as directed.

Second Day

1. Remove cultures from the incubator and observe colony morphology of each organism as it appears on SBA.

2. Record colony size, color, and hemolytic pattern for all sixteen organisms in the group in the appropriate box on the report sheet provided. Allow one entire horizontal row of boxes on the report sheet for each organism. This enables you to keep the respective

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*This mimeo supersedes M 08-280-430, 015

morphological and biochemical characteristics separated from those of other organisms. There should be two different colony types for each specimen.

3. Perform a catalase test on all beta hemolytic organisms in your group only.

4. Perform a bacitracin susceptibility test on all beta hemolytic organisms that are catalase negative. Each student should have two bacitracin tests to perform, so each group of two should share one (1) SBA plate.

5. Incubate the inoculated bacitracin tests in the CO_2 incubator or candle jar.

6. Keep the original culture plates in your area as you will be using them for a gram stain practice.

7. Clean up as directed.

Third Day

1. Remove susceptibility tests from the incubator and primary isolation plates from the storage refrigerator.

2. Interpret the completed susceptibility tests and record the results in the appropriate box on your report sheet.

3. Complete your final report in one of the following ways:

    a. If there was no beta hemolytic organisms on the original plate report "No beta Streptococcus isolated".

    b. If there was beta hemolytic organism present but they were catalase positive, report "No beta Streptococcus isolated"

    c. If you performed a bacitracin test on a specimen and it was resistant report "beta Streptococcus not group A isolated".

    d. If you performed a bacitracin test on a specimen and it was susceptible report "Presumptive group A beta Streptococcus isolated".

4. An instructor will review the results with the class. If you have any discrepancies, discuss them with an instructor at that time.

5. All your cultures should be completed. All culture materials should be discarded in autoclave buckets.

6. Clean-up as directed.

Throat Specimen Report Sheet

Spec. no.	Colony morphology sheep blood agar	Catalase	Bacitracin	Conclusions
A-1				
A-2				
A-3				
A-4				
B-1				
B-2				
B-3				
B-4				

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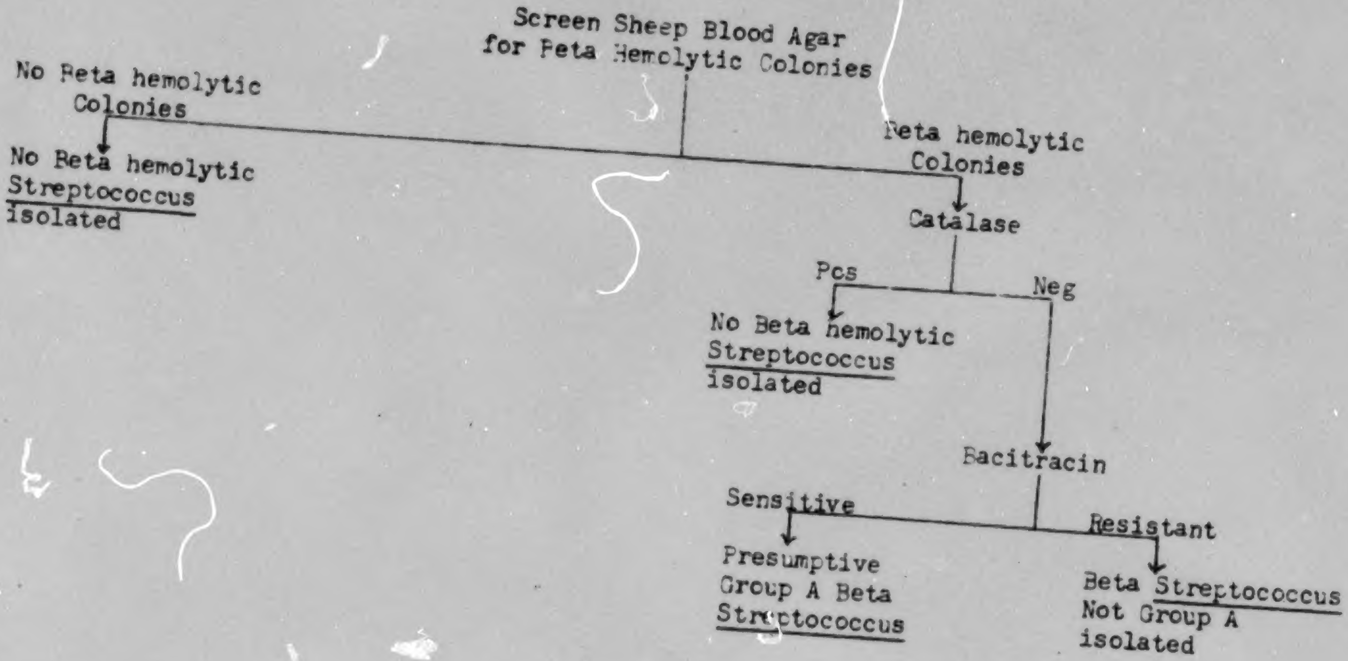
Throat Specimen Flow Chart


Screen Sheep Blood Agar
for Beta Hemolytic Colonies
No Beta hemolytic
Colonies
No Beta hemolytic
Streptococcus
isolated
Beta hemolytic
Colonies
Catalase
Pos
Neg
No Beta hemolytic
Streptococcus
isolated
Bacitracin
Sensitive
Resistant
Presumptive
Group A Beta
Streptococcus
Beta Streptococcus
Not Group A
isolated

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SFC Lyle J. Sorensen

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-417
LABORATORY SCIENCES DIVISION 048
MICROBIOLOGY BRANCH

Sterilization and Decontamination Practical Exercise (5 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Procedures (Basic)

PLACE: Laboratory

REFERENCES: All previous references

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: None

TOOLS, EQUIPMENT, AND MATERIALS: Autoclave, glassware washer, glassware dryer, and cleaning supplies

PERSONNEL: One instructor per 5 students (MOS 92B3H)

INSTRUCTIONAL AIDS: None

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Practical exercise

I. INTRODUCTION (5 min)

A. Opening Statement: This practical exercise will acquaint you with the equipment and methods used in sterilization and decontamination of bacteriological materials.

Bacteriological identification requires that pure cultures of microorganisms be studied. Since microorganisms are ubiquitous, all materials used in the cultivation of bacteria must be subject to preliminary sterilization.

The fundamental principle of media sterilization is to remove or destroy all living material on or within a medium without rendering it ineffective for cultivating the desired microorganisms. The methods most commonly used involve moist or dry heat and filtration. The particular sterilization method employed is governed by the items to be sterilized and their intended use.

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*This lesson plan supersedes LP 08-280-417, 046

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B. Objectives

1. Utilize the autoclave, dishwasher, or other means of sterilization and cleaning to decontaminate and/or prepare materials for disposal or reuse, as demonstrated in class.

C. Class Procedure and Lesson Tie-In

This PE will be performed at appropriate times throughout the bacteriology course.

II. APPLICATION (350 min)

A. Instructors' Activities

1. Instruct students on proper operation of autoclave, glassware washer, and glassware dryer.
2. Explain steps in preparing material and equipment for sterilization.
3. Discuss safety rules to prevent laboratory infections.
4. Inspect laboratory equipment and working areas.
5. Direct students to storage areas for materials and equipment.
6. Maintain classroom control and enforce safety precautions.
7. Supervise clean up.
8. Discuss individual results with students.

B. Students' Activities

1. Operate autoclave, glassware washer, and glassware dryer.
2. Damp mop floors and disinfect table tops before pouring media.
3. Wash glassware in hot water, then in distilled water, and dry in glassware dryer.
4. Clean and decontaminate work area with disinfectant solution.
5. Discard all waste material when analyses have been completed.

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III. CRITIQUE (5 min)

A. Questions From Students

B. Review of Main Points

1. Autoclave operation

2. Importance of sterilization and decontamination

3. Safety factors

C. Closing Statement: The working surfaces of the laboratory should be swabbed with phenol, cresol, or other effective antiseptics before and after working with infectious materials. Work with highly infective and dangerous organisms should be attempted by a technician well trained in bacteriology and having full knowledge of the materials and equipment at his disposal.

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MODIFICATIONS

LP# 08-280-418 of this publication has (have) been deleted in adapting this material for use in Vocational and Technical Education. Deleted material involves extensive use of military forms, procedures, systems, etc. and was not considered appropriate for use in vocational and technical education.

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SFC Lyle J. Sorensen

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-419
LABORATORY SCIENCES DIVISION 058
MICROBIOLOGY BRANCH

Gram Stain Examination (2 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic)

PLACE: Classroom

REFERENCES: All appropriate previous references.

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: See Annex A and M 08-280-419-1 and -2.

PERSONNEL: Eight instructors (MOS 92B3H or 92B4H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, eraser

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Practical Examination

I. INTRODUCTION (5 min)

A. Opening Statement: The function of a bacteriology laboratory technician is to isolate and identify organisms. The Gram stain serves as a valuable adjunct in accomplishing these procedures. This technique will be employed to aid in identifying and reporting the genus of organisms issued during practical exercises.

B. Objective:

Given bacterial specimens in broth, swab, and plate, correctly prepare, stain, and interpret the smear and report the correct Gram morphology for each organism given according to standards stated in the performance evaluation checklist.

C. Class Procedure and Lesson Tie-In

1. The Gram stain graded P.E. is a crucial objective.

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*This lesson plan supersedes LP 08-280-419, 065.

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2. Gram stains will be performed throughout the course and the ability to perform them correctly will affect identification of organisms.

II. EXAMINATION (75 min)

A. Instructors' Activities

1. Day prior to examination exercises

   a. Inoculate plates from stock cultures (see Annex B).

   b. Incubate cultures.

   c. Quality control staining reagents

2. Day of examination

   a. Instruct students to prepare area for Gram stain examination.

   b. Instruct students to pair off and issue culture plates to each pair.

   c. Instruct students to perform Gram stains step by step with instructor observing and approving each step (see Annex C).

   d. Supply report sheets (M 08-280-419-2).

   e. Hand out folders of pre-stained slides to each pair of students.

   f. Instruct students to record the Gram morphology as observed on the five slides.

   g. Instruct students to properly place their stained slides on the microscope and have an instructor check each slide.

   h. Make all appropriate statements, comments, and errors on performance checklist (see M 08-280-419-1 and Annex C).

B. Students' Activities

1. Prepare area for Gram stain exam.

2. Pair off into groups of two.

3. Obtain unknown cultures (two plates per pair).

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4. Obtain a folder of pre-stained slides (two sets of five each).

5. Obtain a report sheet M 08-280-419-2.

6. Follow instructor's directions (see Annex C).

7. Students may be allowed to repeat any slide they are not completely satisfied with, but only once per slide.

8. When not performing preparation of smears or stains, observe the prepared slides and record on the report sheet the gram morphology for each slide.

9. Remember this is a graded exam and no talking will be allowed.

10. After instructor has graded the stains and collected the report sheets, clean the area and place microscopes in the proper area.

III. REVIEW AND RETEACHING (10 min)

A. Questions From Students

B. Review of Gram stain technique with reference to common errors

C. Closing Statement: This P.E. will test your knowledge and technique in Gram staining. It is a valuable means of checking your staining technique and indicating your weak areas. In the field of Bacteriology you will find the Gram stain a valuable tool and the necessity to perform it as well as possible cannot be overemphasized.

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ANNEX A
INSTRUCTIONAL AIDS

Supplies and Equipment

1.	Autoclave, laboratory	1 per class
2.	Refrigerator, 12 to 14 cu. ft.	1 per class
3.	Incubator, biological large	1 per class
4.	Microscope, binocular, complete	1 per student
5.	Loop, inoculating, with platinum or nichrome wire	1 per student
6.	Needle, inoculating, with platinum or nichrome wire	1 per student
7.	Pencil, wax	1 per student
8.	Gram stain reagents	1 set per sink
9.	Lens paper	1 pk per class
10.	Disinfectant, germicidal and fungicidal	2 gallons per class
11.	Detergent, surgical	1 gallon per class
12.	Racks, test tube, metal 40-hole	1 per student
13.	Racks, test tube, metal, 90-hole	1 per student
14.	Burner, gas, laboratory, Bunsen, or Touchamatic	1 per student
15.	Buckets, metal, decontamination	4 per class
16.	Towels, paper	16 packages per class
17.	Slides microscopic, plain 25 x 75 mm.	1 box per student
18.	Immersion oil, microscopic	1 bottle per student
19.	Staining rack (complete)	1 per sink
20.	Gram stain report sheet M 08-280-419-2	1 per student

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Procedures and Materials for Preparation of Cultures

A. Materials

NOTE: Materials and procedure preparation are based on 40 students only.

1. Forty plates of basal medium
2. Stock cultures
    a. *Staphylococcus* species
    b. *Escherichia coli*
3. Felt tip marker
4. Masking tape
5. Burner with tubing
6. Disinfectant
7. Inoculating loop

B. Preparation Procedure

NOTE: Inoculation of media must be done the day prior to Gram stain exam for proper incubation time.

1. Inoculate twenty plates of basal medium with organism (A.2.a.) and twenty plates with organism (A.2.b.). Streak plates with an inoculating loop.
2. Incubate all plate cultures at 37°C for 18-24 hours.

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ANNEX C
INSTRUCTIONAL AIDS

Protocol

A. Materials

1. Forty sets of pre-stained slides

NOTE: Materials are based on a class of forty. The slides will be arranged in booklets, two sets of slides per booklet, each set containing five pre-stained slides.

   a. Description of slides

      (1) Gram positive cocci

      (2) Gram positive bacilli

      (3) Gram negative cocci

      (4) Gram negative bacilli

      (5) Gram positive cocci and Gram negative bacilli

2. Forty plates of cultures

   a. Twenty plates streaked with a Staphylococcus species.

   b. Twenty plates streaked with Enterobacter species.

   c. Check plates for pure culture.

3. Student report sheets M 08-280-419-2.

4. Instructor's checklist M 08-280-419-1.

B. Procedure for Gram Stain Exam

1. Instructions given to class

   a. No talking during exam and each individual will perform his own staining.

   b. Students prepare slides from growth on the two plates provided.

   c. Students interpret five pre-stained slides.

   d. Crucial exam - 90 percent accuracy

e. Time limit two hours

2. Issue five pre-stained slides

    a. Booklet of ten slides to each pair of students

    b. Each slide is keyed.

    c. Each student works on his/her five slides, interpreting and reporting the Gram morphology on M 08-280-419-2.

3. Issue plate cultures

    a. Each pair of students receives two plate cultures.

    b. Each student will prepare one slide from each plate culture.

    c. Instructor observes students in small groups (four at a time) and indicates on the checklist any errors observed.

    d. Students prepare smears, let air dry.

    e. Students heat-fix slides.

NOTE: While slides are drying for one group of students, instructor may move on to another group and assist them in getting started.

    f. Students go to designated sinks and stain the slides.

    g. Students observe slides, then leave each slide in focus until instructor observes slides.

    h. Students' slides must follow criteria under C.3.a.(5.) (readable smear) in Annex C.

    i. Allow only one repeat of each slide.

    j. After students complete examination, collect report sheets and grade.

C. Gram Stain Examination Checklist

1. For instructor's use in grading the students in Gram stain examination.

2. Consists of two main reactions

   a. Performance

      (1) Students' ability to prepare, fix, and stain smears.

      (2) Students' ability to properly use microscope.

   b. Interpretation

      (1) Students observe pre-stained slides.

      (2) Students' ability to correctly interpret and report slides.

3. Detailed description of checklist use.

   a. Performance

      (1) Smear preparation

         (a) Label slides with culture number.

         (b) Use loop to transfer water to slide.

         (c) Use needle to transfer organism to slide.

         (d) Make emulsion and spread it evenly (about a half-inch in size).

         (e) Let smear air dry.

      (2) Heat fixing

         (a) Pass slide through flame rapidly, smear side up.

         (b) Do not scorch slide.

      (3) Staining

         (a) Use all stains in correct order.

         (b) Time limits followed (at least the minimum).

         (c) Decolorization procedure followed as taught in class.

         (d) Slides must be positioned smear side up during staining procedure.

(e) Slides may be blotted dry or air dried.

(4) Microscope use

(a) Student properly focuses slide into view.

(b) Oil immersion lens used.

(c) Oil under objective.

(d) Slide is smear side up.

(e) Proper use of light source.

(5) Readable smear

(a) Organisms are present and visible.

(b) Cells not distorted by overheat-fixing or other student errors.

(c) Gram reaction of slide corresponds to that of key.

(d) Smear is not so thick as to make identification of shape or actual arrangement of cells indistinguishable.

b. Interpretation

(1) Students report complete Gram morphology for each slide to include Gram reaction, shape, and arrangement of cells.

(2) Students' Gram morphology agrees with that of key.

c. Grading errors

(1) Any failure of the criteria listed under Performance or Interpretation is considered an error and is indicated by a check in the appropriate block.

(2) Grading of both performance and interpretation is done after the examination; however, performance is observed and recorded during exam for errors.

(3) Any check made in either performance or interpretation constitutes a failure.

SSG Dennis Dougherty

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-420
LABORATORY SCIENCES DIVISION 048
MICROBIOLOGY BRANCH

Classification of Gram Negative Bacilli (1 hour)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES:

Bailey, W.R., and Scott, E.G. Diagnostic Microbiology. 4th ed. St. Louis: C. V. Mosby Company, 1974, chap 20 and 21.
Lennette, E. H. & others (ed), Manual of Clinical Microbiology.2nd ed. Washington: American Society for Microbiology, 1974, chap 18, 23,& 24
Bodily, H.L. *et al*. Diagnostic Procedures. 5th ed. New York: American Public Health Association, 1970, chap 11.

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: Not applicable

PERSONNEL: One primary instructor (MOS 92 B3H)

INSTRUCTIONAL AIDS: Chalk, magnetic chalkboard, and eraser

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Lecture

I. INTRODUCTION (5 min)

A. Opening Statement: Although the gram negative bacilli are composed of many bacterial species exhibiting various pathogenic and cultural characteristics, an understanding of the gram gram negative bacilli is not difficult if you master the basic information presented during this period. By utilizing this information in a medical laboratory, you will be able to identify organisms which cause such diseases as typhoid fever, dysentery, and pneumonia.

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*This lesson plan supersedes LP 08-280-420, 036

B. Objective: Given a list of characteristics and identification methods of the family Enterobacteriaceae; non-enteric gram negative bacilli; and fastidious gram negative bacilli, select characteristics or identification methods used in the identification of those bacteria.

C. Class Procedure and Lesson Tie-in: A practical exercise with known gram negative bacilli follows this lecture.

II. EXPLANATION (30 min)

A. Classification of Gram Negative Bacilli

1. Family Enterobacteriaceae

2. Non-enteric gram negative bacilli

3. Fastidious gram negative bacilli

B. Major Characteristics

1. Family Enterobacteriaceae

a. Growth characteristics

(1) Grows well on basal medium

(2) Incubation 18-24 hours at 37 C

(3) Colony morphology on sheep blood agar

(a) Routine

1 Mucoid to grey

2 Medium to large

3 Gamma or beta hemolysis

(b) Swarming

1 Caused by highly motile organism--Proteus species

2 Report as swarming

b. Gram morphology

(1) Gram negative

(2) Bacilli

(3) No predominant arrangement

c. Glucose metabolism

(1) Ferments glucose rapidly, producing acid end-products

(2) May or may not produce gas (aerogenic and anaerogenic)

d. Oxidase negative

e. Reduces nitrates to nitrites ($NO_3 \dashrightarrow NO_2$)

f. Pathogenicity

(1) Normal flora in intestinal tract

(2) Three enteric pathogens of intestinal tract in adults

(a) *Salmonella* species

(b) *Shigella* species

(c) *Arizona* species

(3) Usually pathogenic outside of the intestinal tract

(4) Some *Escherichia coli* are pathogenic in infants

2. Non-enteric gram negative bacilli

a. Growth characteristics

(1) Grow well on basal medium

(2) Incubation 18 to 24 hours at 37 C

(3) Colony morphology on sheep blood agar

(a) Mucoid to gray

(b) Medium to large

(c) Gamma to beta hemolysis

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(4) Special traits

(a) Pseudomonas species may produce a blue-green pigment on various media

(b) Pseudomonas species may produce a characteristic grape-like odor

b. Gram morphology is the same as the family Enterobacteriaceae

c. Glucose is not fermented

d. Most are oxidase positive

c. Pathogenicity

(1) Possible pathogens in most specimens

(2) Secondary invader

3. Fastidious gram neagtive bacilli

a. Requires basal medium plus enrichments for growth

b. Will not grow on MacConkey or Hektoen agar

c. Grows well on chocolate agar

d. Gram morphology

(1) Gram negative

(2) Bacilli or cocco-bacilli

(3) No predominant arrangement

C. General Methods for Identification

1. Enterobacteriaceae and non-enteric gram negative bacilli

a. Primary identification by carbohydrate utilization

b. Routine identification by established biochemical patterns

c. Identification of enteric pathogens by serological typing

(1) Salmonella species and Shigella species

(2) Enteropathogenic Escherichia coli (EEC)

2. Fastidious gram negative bacilli require special techniques for identification

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D. Questions from Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Classification

2. Major charcteristics

3. General methods for identification

B. Closing Statement: Certain gram negative bacilli can incapacitate infected individuals to the extent that an entire Army unit may become completely ineffective. If you can isolate and identify the causative agent, you may contribute to the recovery of affected patients and the eventual elimination of the source of infection.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
MICROBIOLOGY BRANCH

*IM 08-280-420
048

Classification of Gram Negative Bacilli

Although the family Enterobacteriaceae is composed of many bacterial species exhibiting various pathogenic and cultural characteristics, and understanding of the Enterobacteriaceae is not difficult if you master the basic information presented during this period. By utilizing this information in a medical bacteriology laboratory, you will be able to isolate and identify organisms which cause such diseases as typhoid fever, dysentery, and pneumonia.

Organisms classified as members of the family Enterobacteriaceae are commonly referred to as enteric bacteria, since they are found in the intestinal tract of man an animals. In this area of the body most enteric organisms are normal flora; others are pathogenic parasites. Since enterics normally inhabit the gastrointestinal tract, it is obvious that they are easily distributed to other environmental areas such as soil and water. Certain criteria must be met for an organism to be listed in this family. All are gram-negative bacilli which attack and ferment glucose within 24 hours; the end products of this fermentation contribute to a strong acid pH. Most members also produce gas (CO_2 and/or H_2) as a result of glucose degradation, and are referred to as aerogenic. Family members which produce no gas when attacking glucose are described as anaerogenic. All members reduce nitrates to nitrites; are oxidase negative; catalase positive; and grow readily on both basal and enriched media. Any gram-negative bacillus which is found in the intestinal tract and ferments glucose rapidly with or without the production of gas meets the major criteria for classification as a member of the family Enterobacteriaceae.

Lactose utilization divides enteric organisms into three broad groups. Some attack lactose rapidly (within 24 hours) while others are delayed in their ability to break down lactose but may effect this change within 48 to 72 hours. They are referred to as late lactose fermenters. Enteric bacteria which do not attack lactose are called nonlactose fermenters. While grouping on the basis of a reaction to lactose is the first step, it is by no means adequate for identification. The major groups of enteric organisms are not classified by lactose utilization alone, but upon a variety of biochemical reactions, since organism within a major group often exhibit different properties when tested for lactose fermentation.

Other biochemical properties tested include hydrogen sulfide production, urease production, indole production, citrate utilization, malonate utilization, phenylalanie deaminase and lysine decarboxylase production, and carbohydrate fermentation patterns. The principles and methods employed for these procedures will be discussed later. Many additional biochemical tests are available. Often the choice of one particular test is based upon a combination of capability and personal choice but some tests, such as urease production,

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*This instructors manuscript supersedes IM 08-280-420, 036 are universal. The test battery for identification used in this course is adequate for routine laboratory work in processing enteric organisms.

Enteric bacteria are also identified serologically. This is known as serological typing and is basically similar to the slide and tube typing procedures used in immunohematology. However, bacteria are used as the antigen in place of red cells and the antisera are mixed with suspensions of the unknown organisms on either a slide or in a tube. Agglutination occurs when the antigen is mixed with specific antisera because, just as with red blood cells, bacteria contain several types of antigens. The primary antigens are located in the bacterial cell wall; they are called somatic or "O" antigens and are heat stable. Many types of antigens whic can be extracted from a bacterial cell wall are used to produce polyvalent antisera in an animal such as a rabbit. These polyvalent antisera are used to identify many different antigens in a group, such as those possessed by the various species of Shigella. Organisms possessing a capsule have an envelope or "K" antigen as part of the capsular material. This antigen can be eliminated by heating. A well known "K" antigen is possessed by Salmonella typhi designated with the symbol "Vi". Motile organisms possess flagellar antigens designated as "H" antigens, which are also thermolabile. Antisera specific for these antigens are used primarily for identifying serotypes of Salmonella. Type specific diagnostic sera is used for confirmation of enteropathogenic Escherichia coli. Serological typing is generally used to confirm the identity of an organism established through biochemical patterns. At times the methodology is reversed; biochemical patterns confirm serological typing.

Several different gram-negative bacilli may be associated and isolated with enteric organisms. These differ morphologically and biochemically from enteric bacilli. All are nonfermetative; many are oxidase positive. Pseudomonas species may produce water soluble green and blue-green pigments; produce a pellicle when grown in liquid media; produce a metallic sheen on TSI; and produce cytochrome oxidase. Some species of Acinetobacter do not produce cytochrome oxidase, while others are nonmotile and do not utilize citrate whereas Pseudomonas species metabolize citrate as a sole carbon source and are found in other areas of the body they may exhibit varying degres of pathogenicity.

The enteric bacteria are studied because they consitute the predominant organisms in the intestinal tract of man. Since their normal habitat is the intestinal tract, they are routinely isolated from fecal specimens in the form of stools or rectal swabs. The saprophytic enterics which constitute the normal flora must be distinguished from pathogenic enterics which cause intestinal disease. If an enteric organism gains entry to an area of the body other than the intestinal tract, such as the abdominal cavity, urinary tract, or lungs, it may be either the primary or secondary agent of a disease process in that specific site. For this reason enteric organism isolated from specimens other than stools or rectal swabs are identified as to genus and species with antibiotic susceptibility studies performed.

The major characteristics to be considered when studying the enteric bacteria include habitat, gram morphology, and fermentation of glucose. Certain groups of enteric bacteria cause intestinal disease; other groups constitute the normal flora of the intestinal tract. All members of the family Enterobacteriaceae are potential pathogens when introduced to other areas of the body. Identifying organisms according to their genus and species requires consideration of lactose utilization; determination of their biochemical pattern, and performance of serological studies. Gram-negative bacilli not classified in the enteric group include Pseudomonas, Proteus, and Acinetobacter species. Organisms of these three genera are frequently isolated from sources which also yield enteric bacteria.

Certain enteric organisms can incapacitate infected individuals to the extent that an entire Army unit may become completely ineffective. If you can isolate and identify the causative agent, you can contribute to the recovery of affected patients and the eventual elimination of the source of infection.

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*LP 08-280-421
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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
MICROBIOLOGY BRANCH

Stool Specimen Practical Exercise (6 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic)

PLACE: Classroom

REFERENCES: All previous references

STUDY ASSIGNMENT: M 08-280-421-1, read before class

STUDENT UNIFORM AND EQUIPMENT: M 08-280-421-1

TOOLS, EQUIPMENT, AND MATERIALS: See annexes A - C

PERSONNEL: Eight instructors (MOS 92B3H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, and eraser; TV receivers

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Practical exercise

1. INTRODUCTION (5 min)

   A. Opening Statement: You will be required to work with bacteria isolated from clinical specimens during this exercise, throughout this course, and when assigned to clinical laboratories. This exercise is designed to give you practice performing procedures routinely employed for isolation and identification of enteric organisms studied.

   B. Objective

   Given simulated stool specimens, and all necessary materials, inoculate media, interpret and report colony morphology and reactions of biochemical tests used in the identification of intestinal pathogens, as demonstrated in class.

   C. Class Procedure and Lesson Tie-in

      1. Application of information received during previous lectures and demonstrations.

      2. Practical exercise with known species of Escherichia, Klebsiella, Proteus, Providencia, Arizona, Citrobacter, Pseudomonas, Shigella,

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*This lesson plan supersedes LP 08-280-421, 016

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and Salmonella is conducted for a total of 6 hours during 4 consecutive days with the last hour as review of the entire P.E. Specific tasks assigned by the instructor during each phase will lead to completion of the exercise by the final hour.

II. APPLICATION (235 min)

A. Instructors' Activities

1. Class divided into two-person teams
2. Equipment and materials issued (See Annex A & B)
3. Pure cultures prepared and issued (See Annex C & D)
4. Daily instructions given
5. Questions answered
6. Corrections made
7. Laboratory safety maintained
8. Classroom control exercised

B. Students' Activities

1. Aseptic technique maintained
2. Appropriate media inoculated and streaked for isolation (See M 08-280-421-1)
3. Colony morphology observed and recorded (See M 08-280-421-1)
4. Biochemical tests performed (See M 08-280-421-1)
5. Culture materials discarded
6. Clean up performed as directed
7. Equipment stored

C. Organization of Instructional Hours

1. First Day (35 min)

a. Each pair of students receives a lettered rack (A & B), which contains 8 specimens of enteric bacteria, 4 of each series (A & B). The specimens are arranged so that each two-student group works with bacteria that give all the various biochemical reactions studied.

b. Inoculate, streak for isolation, and incubate media according to M 08-280-421-1.

c. Inoculate one sheep blood agar plate according to M 08-280-421-1.

d. Specimens discarded

e. Clean up as directed.

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2. Second Day (80 min)

   a. Colony morphology studied and recorded (See M 08-280-421-1)

   b. Inoculate TSI for each specimen that may be pathogenic. (See M 08-280-421-1)

NOTE: TSI's should have a generous butt and a well formed slant to insure proper results. TSI's should be inoculated from plates that show the best isolation and organism differentiation.

   c. Incubate TSI's

   d. Cultures from first day refrigerated

   e. Clean up as directed

3. Third Day (80 min)

   a. TSI's studied and patterns recorded

   b. Inoculate key series of biochemicals as directed by M 08-280-421-1.

   c. Show videotape on inoculation of enteric biochemicals

NOTE: Number or label tubes on the tube itself, and not the caps, with a wax pencil.

   d. Biochemicals incubated along with TSI's of previous day

   e. Clean up as directed

4. Fourth Day (30 min)

   a. Interpret key biochemicals and record results

   b. Compare key biochemicals with appropriate TSI patterns and identify organisms

   c. All culture material discarded

   d. Clean up as directed

III. REVIEW (10 min)

A. Review of Main Points

1. Inoculating and streaking technique

2. Lactose fermentation patterns

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3. Inoculation and interpretation of TSI

4. Inoculation and interpretation of biochemicals

5. Identifying organisms by TSI and key biochemicals

B. Closing Statement: Knowledge, however broad, of theories and principles of clinical bacteriology is important but it must be correctly channeled into medical laboratory tasks. You must develop the ability to apply your knowledge in a planned, orderly manner so that you can efficiently perform your duties as a clinical bacteriology specialist.

ANNEX A
INSTRUCTIONAL AIDS

Supplies and Equipment

1. Autoclave, laboratory (1 per class)
2. Refrigerator, large (1 per class)
3. Incubator, large (1 per class)
4. Loop, inoculating (1 per student)
5. Needle, inoculating (1 per student)
6. Pencil, wax (1 per student)
7. Swabs, sterile in containers (6 per student)
8. Disinfectant, germicidal (2 gallons per class)
9. Detergent, surgical (1 gallon per class)
10. Racks, test tube, metal, 40-hole (1 per student)
11. Racks, test tube, metal, 90-hole (1 per student)
12. Burner, gas, touchamatic (1 per student)
13. Buckets, metal, decontamination (4 per class)
14. Towels, paper (1 box per student)
15. Tape, masking (3 rolls per class)
16. Canister, plate, metal (1 per student)

ANNEX B
INSTRUCTIONAL AIDS

Media and Reagents

1.	Sheep blood agar (SBA)	1 plate per student
2.	Hektoen agar (HEK)	5 plates per student
3.	MacConkey agar (MAC)	4 plates per student
4.	Triple sugar iron agar (TSI)*	6 tubes per student
5.	Indole*	1 tube per student
6.	Motility*	2 tubes per student
7.	Urease*	1.5 tubes per student
8.	Lysine decarboxylase*	1.5 tubes per student
9.	Phenylalanine deaminase*	2 tubes per student
10.	ONPG	3.5 tubes per student
11.	CN Hajna	1 tube per student
12.	Kovac's reagent	1 bottle per four students
13.	Ten percent ferric chloride solution	1 bottle per four students

NOTE: All plate media - 15 ml medium per plate; TSI, citrate, phenylalaine - 4 to 5 ml per tube and slanted; all other tubed media - 3 to 5 ml per tube unless indicated.

*13 x 100 mm test tubes biochemical test medium.

ANNEX C
INSTRUCTIONAL AIDS

Stock Cultures Required

1. *Escherichia coli*
2. *Citrobacter* species (RLF)
3. *Salmonella* species (H_2S pos)
4. *Salmonella* species (H_2S neg)
5. *Proteus mirabilis*
6. *Klebsiella* species
7. *Arizona* species
8. *Providencia* species
9. *Shigella* species
10. *Proteus vulgaris*
11. *Pseudomonas aeruginosa*

NOTE: All cultures inoculated to 100 ml of trypticase soy broth or Todd-Hewitt broth day prior to P.E., then incubated. Day of practical exercise, prepare mixtures of organisms, dip swabs into appropriate mixtures, and dispense according to Annex D.

ANNEX D
INSTRUCTIONAL AIDS

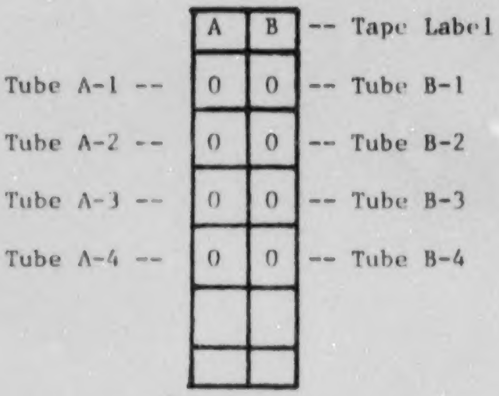
Protocol

Mixtures for Specimens

GROUP A
1. *Escherichia coli* and *Proteus mirabilis* (infant stool)
2. *Salmonella* species (H_2S pos) and *Klebsiella* species
3. *Proteus Vulgaris* and *Shigella* species
4. *Providencia* species and *Citrobacter* species (RLF)

GROUP B
1. *Klebsiella* species and *Pseudomonas* species (infant stool)
2. *Escherichia coli* and *Shigella* species
3. *Arizona* species and *Proteus Vulgaris*
4. *Citrobacter* species (RLF) and *Salmonella* species (H_2S neg)

DIAGRAM


A
B
-- Tape Label
Tube A-1 --
0
0
-- Tube B-1
Tube A-2 --
0
0
-- Tube B-2
Tube A-3 --
0
0
-- Tube B-3
Tube A-4 --
0
0
-- Tube B-4

NOTE: One rack arranged in this manner is needed for every two students. Care should be taken to arrange organisms exactly as indicated so that they will match the students instruction sheet (M 08-280-421-1).

SSG Dennis Dougherty

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-423
LABORATORY SCIENCES DIVISION 048
MICROBIOLOGY BRANCH

Enteric Media - 1 hour

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES:

Baily, W. R., and Scott, E. G. Diagnostic Microbiology. 3rd ed. St. Louis: C. V. Mosby Company, 1974, chapter 39

Baltimore Biological Laboratories. BBL Manual of Products and Laboratory Procedures. 5th ed. Cookeysville: BBL Division of BioQuest, 1973.

Lennette, E. H., and others (eds). Manual of Clinical Microbiology. 2nd ed. Washington: American Society for Microbiology, 1970, chap 95.& 96

Difco Laboratories, Inc., Difco Manual of Dehydrated Culture Media and Reagents for Microbiological and Clinical Laboratory Procedures. 9th ed. Detroit: Difco Laboratories, Inc., 1971.

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: Not applicable.

TOOLS, EQUIPMENT, AND MATERIALS: Not applicable.

PERSONNEL: One instructor, MOS 92B30

INSTRUCTIONAL AIDS: Chalk, magnetic chalkboard, and eraser; MP 08-280-423-1 thru- 10 (see Annex A)

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Lecture

I. INTRODUCTION (5 min)

A. Opening Statement: The purpose of employing enteric media is twofold: to recover enteric pathogens from rectal swabs and stool specimens and to accelerate isolation of enteric organisms from clinical specimens such as wounds, sputum, and urine. On the basis of growth patterns and metabolic reactions, you may group bacteria from a given specimen into categories of rapid

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*This lesson plan supersedes LP 08-280-423, 026

lactose fermenters, late lactose fermenters, or nonlactose fermenters. This grouping aids in determining further procedures to follow for species identification.

B. Objectives:

1. Given an enteric medium, select the correct classification for that medium.

2. Given a description of bacterial growth on an enteric medium, select the correct reporting terminology for that growth.

3. From a list of bacteria, select the bacteria that will grow and those that are inhibited by various enteric media.

C. Class Procedure and Lesson Tie-in

1. Practical exercise using enteric media follows for reinforcement of lecture material.

2. Unknown practical exercises will be conducted during ensuing weeks to improve proficiency in using enteric media to isolate and identify enteric bacteria.

II. EXPLANATION (35 min)

A. Purpose of Enteric Media

1. Demonstrate enteric pathogens in fecal specimens

2. Isolation of gram negative bacilli from any clinical specimen

B. Classification of Enteric Media

1. Enrichment

   a. Definition: a liquid medium used to enhance the growth of enteric pathogens while retarding the growth of non-pathogens.

   b. Examples

      (1) Gram negative broth--GN Hajna

      (2) Selenite F broth

2. Selective

- a. Purpose: a plating medium which selects out certain organisms for optimum growth, and contains an indicator which differentiates those selected organism on the basis of their metabolic differences.
- b. Examples
  - (1) MacConkey
  - (2) Hektoen

C. Gram negative Broth (GN Hajna)

- 1. Inhibitors
  - a. Sodium desoxycholate
  - b. Sodium citrate
  - c. Mannitol
- 2. Inhibits the growth of
  - a. Gram positive cocci and bacilli
  - b. Gram negative cocci
  - c. Most rapid lactose fermenting organisms
  - d. Proteus species
- 3. Enhances the recovery of
  - a. Salmonella species
  - b. Shigella species
- 4. Use
  - a. Specimens
    - (1) Stool specimen
    - (2) Rectal swab
  - b. Inoculate specimen directly to medium
  - c. Incubate 6-8 hours at 37 C

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d. Subculture to enteric plating media after incubation

NOTE: Do not incubate for more than 18 hours

QUESTION: When culturing for *Salmonella* and *Shigella* species, why is an enrichment broth, such as GN Hajna, used in addition to direct streaking of clinical specimens?

ANSWER: Fecal specimens contain large numbers of saprophytic enteric bacteria capable of overgrowing any *Salmonella* or *Shigella* species present. The broth changes the population ratio to favor isolation of the enteric pathogens, although they were initially present in small numbers

D. MacConkey Agar (MAC)

1. Basic ingredients
    - a. Nutrients
        - (1) Basal medium--proteins
        - (2) Carbohydrate--lactose
    - b. Inhibitors
        - (1) Bile salts
        - (2) Crystal violet
    - c. Inhibits the growth of
        - (1) Gram positive bacilli and cocci
        - (2) Gram negative cocci
    - d. pH indicator--neutral red
        - (1) In alkaline pH demostrates a colorless appearance
        - (2) In acid pH demonstrates a pink to red color
2. Interpretation of results after 18-24 hours at 37 C
    - a. Pink to red colony--rapid lactose fermenter(RLF)
    - b. Colorless colony--non-lactose fermenter (NLF)

NOTE: Some rapid lactose fermenting organisms will use the lactose in the heavily inoculated area before the routine incubation period has elapsed. When this happens the bacteria will then utilize the protiens in the basal medium as a source of nutrients. At that time the pH of the bacterial colonies in the heavily inoculated area will revert to alkaline producing colorless to yellow colonies in the heavy growth area and pink colonies in the isolated area. This type of bacterial growth is called reversion.

3. Reporting of colony growth
    - a. Pink to red colonies: report as RLF
    - b. Colorless colonies: report as NLF
    - c. Reversion: report as RLF

E. Hektoen (HEK)

1. Basic ingredients
    - a. Nutrients--basal medium
    - b. Carbohydrates
        - (1) Lactose
        - (2) Salicin
        - (3) Sucrose
    - c. Inhibitor--bile salts
    - d. Inhibits the growth of
        - (1) Gram positive cocci and bacilli
        - (2) Gram negative cocci
    - e. pH inicators
        - (1) Brom thymol blue
        - (2) Acid fuchsin

B. Closing Statement: Your tasks in the clinical bacteriology laboratory will be easier if you are able to intelligently use the special types of media available. The most important consideration is that correct use results in improved patient care.

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ANNEX A
INSTRUCTIONAL AIDS

Magnetic Placards

MP 08-280-423-1	ENTERIC MEDIA
MP 08-280-423-2	PURPOSE
MP 08-280-423-3	CLASSIFICATION
MP 08-280-423-4	ENRICHMENT
MP 08-280-423-5	SELECTIVE
MP 08-280-423-6	MacCONKEY
MP 08-280-423-7	BASIC INGREDIENTS
MP 08-280-423-8	INTERPRETATION
MP 08-280-423-9	HEKTOEN
MP 08-280-423-10	GRAM NEGATIVE BROTH

NOTE: Use MP's where appropriate to explanation.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCE DIVISION
MICROBIOLOGY BRANCH

LP 08-280-424
038

TRIPLE SUGAR IRON AGAR- TSI (1 hour)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES:

Bailey, W. R. and Scott, E. G. Diagnostic Microbiology. 5th ed. St. Louis: The C. V. Mosby Company, 1974, Chap 39.
Baltimore Biological Laboratories, BBL Manual of Products and Laboratory Procedures. 5th ed. Cookeysville: BBL Division of BioQuest, 1973.
Lennette, E. H., etal. Manual of Clinical Microscopy. 2nd ed. Washington: American Society for Microbiology, 1970, Chap 95.
Difco Laboratories, Inc., Difco Manual. 9th ed. Detroit: Difco Laboratories, Inc., 1971, pp 165-166.

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: None

PERSONNEL: One primary instructor (92B3H)

INSTRUCTIONAL AIDS: Chalks, magnetic chalkboard, and eraser; TV 514(see Annex A) and receivers; magnetic placards (see Annex B): T 08-280-424-1 (see Annex C).

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

I. INTRODUCTION: (5 min)

A. Opening Statement: The basis for identifying enteric bacteria includes a systematic study of their biochemical properties. Triple sugar iron agar, known as TSI, is used initially to subdivide the enteric organisms into principal groups on the basis of specific biochemical reactions.

B. Objectives:

1. Given a description of a properly inoculated and incubated TSI, select the correct reporting terminology.

This Lesson Plan supersedes LP 08-280-424, 036

2. Given a list of pathogenic Enterobacteriaceae, select the TSI reaction (s) that may be demonstrated by those bacteria.

3. From the material presented in lecture, select the proper inoculation, and incubation procedure for TSI.

C. Class Procedure and Lesson Tie-in.

1. Practical exercise with known TSI reactions follows for reinforcement of lecture material.

2. Practical exercises using unknown organisms will be conducted during ensuing weeks to improve proficiency in using TSI reactions to identify enteric bacteria.

II. EXPLANATION (35 min)

A. Classification-Description-Inoculation

1. Differential

2. Tubed

3. Slanted

a. Butt for anaerobic metabolism

b. Slant for aerobic metabolism

4. Inoculation

a. Inoculate from one colony on MAC or HEK agar.

(1) Stab butt

(2) Streak slant

b. Incubate 18 to 24 hours at 37 C

NOTE: TSI is not incubated in CO_2 because the pH indicator will be affected and may cause false results.

B. Differential Ingredients

1. Carbohydrates

a. Glucose-- 0.1 percent

b. Lactose-- 1.0 percent

c. Sucrose-- 1.0 percent

2. pH indicator -- phenol red

a. Alkaline -- red

b. Acid -- yellow

3. H_2S indicator system- Produces visible black precipitate

4. Inhibitors - none

C. Biochemical Reactions on TSI -- Enteric Organisms

1. Ingredients utilization

a. Carbohydrates - acid end-products (yellow color)

b. Proteins - alkaline end-products (red color)

2. Production of gas from carbohydrate fermentation

a. CO_2 and/or H_2

b. Not produced by all enterics

c. Indicated by splitting, lifting, and/or bubbling of medium

3. Production of H_2S

a. Blackening of medium

b. Seen in butt-masks yellow color

c. May also be seen on slant

D. Interpretation of TSI Patterns - 18 to 24 hours at 37 C

1. Red slant/yellow butt

a. Written as alkaline/acid (K/A)

b. Gas production (if seen) K/Ag

2. Red slant/black or yellow with black butt

a. Written as alkaline/acid with H_2S (K/A_{H_2S})

b. Gas production (if seen) K/A H_2Sg

3. Yellow slant/yellow butt

a. Written as acid/acid (A/A)

b. Gas production usually seen (A/Ag)

c. Reversion

(1) Top portion of slant is red

(2) Seen only with A/A TSI pattern

(3) Written as (A^R /A)

4. Yellow slant/black or yellow with black butt

a. Written as acid/acid with H_2S (A/AH_2S)

b. Gas production (if seen) A/AH_2Sg

5. Red/orange or no change

a. Written as alkaline/no change (K/NC)

b. No carbohydrates fermented

c. No gas production

d. Non-enteric organism

E. TSI Reactions of Enteric Pathogens

1. Alkaline/Acid (K/A) -- *Shigella* species

2. Alkaline/Acid H_2S (K/A H_2S) -- *Salmonella* species

F. Videotape

1. Introduction

a. Inoculation and interpretation of TSI

b. Points to be observed

(1) Criteria for selecting a good TSI tube

(2) Proper procedure for inoculating TSI

(3) Incubating requirements

(4) Different examples of TSI reactions and patterns

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2. Showing of videotape (TV No. 514)

3. Discussion and questions

III. SUMMARY (5 min)

A. Review of Main Points

1. Classification

2. Ingredients

3. Biochemical reactions

4. Interpretation of TSI patterns

5. TSI reactions of enteric pathogens

6. Videotape

B. Closing Statement: Use and correct interpretation of triple sugar iron agar will enable you to initiate accurate identification procedures on enteric bacteria.

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Videotape

TV No 514, 8.21 minutes. Inoculation and Interpretation of TSI.

This videotape presents the purpose of using TSI. The students are shown properly and improperly prepared tubes of TSI; it is stressed that a tube of TSI to be used for inoculation must have a well formed slant and a generous butt. The proper method of inoculation is shown. The time and temperature of incubation are reinforced. The interpretations of the TSI patterns are demonstrated. Each pattern is interpreted by describing which carbohydrates have been fermented: the color of the slant and the butt portion of the tube: the pH indicated by the color: and the method of recording the TSI pattern. Gas (CO_2 and H_2) production is exhibited by three separate tubes which shows bubbles, splitting of the media, and lifting of the media from the bottom of the tube. H_2S production is shown by the presence of a black precipitate in the butt of the tube. The reason H_2S is formed only in the butt portion of the tube is explained.

NOTE: This videotape is shown during Part II F 2.

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ANNEX B
INSTRUCTIONAL AIDS

Magnetic Placards

1. Classification-Description-Inoculation
2. Differential Ingredients
3. Carbohydrates
4. pH Indicator
5. H_2S Indicator
6. Inhibitors
7. Biochemical Reactions
8. Glucose Fermentation
9. Glucose and Lactose Fermentation
10. Glucose and Sucrose Fermentation
11. Production of H_2S
12. Interpretation of TSI Patterns
13. Glucose Only
14. Glucose and Lactose
15. Glucose and Sucrose, H_2S
16. Glucose and Lactose, H_2S
17. No Carbohydrates Utilized

NOTE: These placards are used as main headings throughout the lecture.

Transparency

T 08-280-424	This transparency is a color representation of the patterns of growth in TSI after inoculation and incubation for 18 to 24 hours.

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LABORATORY SCIENCES DIVISION 038
MICROBIOLOGY BRANCH

Tubed Differential Medium--TSI

The basis for identifying enteric bacteria includes a systematic study of their biochemical properties. Triple sugar iron agar, known as TSI, is used initially to subdivide the enteric organisms into principal groups on the basis of specific biochemical reactions.

Triple sugar iron agar is classified as a differential medium. It is tubed and slanted to form a deep butt for anaerobic metabolism and a slant for aerobic metabolism of its ingredients. The TSI tube is inoculated by first stabbing the butt and then streaking the slant. The inoculated tube is incubated for 18 to 24 hours at 37° C.

Metabolism of the various differential ingredients allows grouping of the Enterobacteriaceae according to certain characteristic reactions. Glucose in 0.1 percent concentration, lactose in 1.0 percent concentration, and sucrose in 1.0 percent concentration may or may not be metabolized by bacteria. Fermentation is used as a means of differentation. Carbohydrate fermentation results in the production of acid end products which are demonstrated by a change in the pH indicator included in this medium. Phenol red, used in TSI, is yellow in an acidic and red in an alkaline environment. Another system present for differentiation is the hydrogen sulfide indicator group: ferrous ammonium sulfate and sodium thiosulfate. Both compounds must be present since the end product, ferrous sulfide, results from a two-step reaction. Certain enteric organisms react with sodium thiosulfate to produce hydrogen sulfide, a colorless gas. The H_2S in turn reacts with the heavy salt ferrous ammonium sulfate to give a visible reaction, a black precipitate of ferrous sulfide. This black precipitate forms in the butt of the TSI tube.

Since TSI medium lacks inhibitory compounds, organisms other than enteric bacteria grow and often show metabolic activity. The reactions are meaningful only for enteric bacteria and related gram-negative bacilli. TSI should be inoculated with gram negative bacilli which have been cultivated on enteric plating media to insure accurate reactions.

There are five significant reactions which occur when the various species of enteric bacteria metabolize the constituents in TSI. They are glucose fermentation glucose and lactose fermentation glucose and sucrose fermentation production of gas from the fermentation of the carbohydrates; and production of hydrogen sulfide. Different species produce varying combination of these five reactions, enabling us to divide the Enterobacteriaceae into several distinct groups.

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*This instructors's manuscript supersedes IM 08-280-424, 036

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After 18 to 24 hours incubation at 37° C, the metabolic activity of the organisms which have been inoculated to tubes of TSI is interpreted and recorded. Certain enteric bacteria are capable of fermenting glucose only. As the 0.1 percent glucose is being utilized both the slant and butt are yellow due to organic acids formed as a result of glucose degradation. The small amount of glucose is depleted before 18 hours. Since the organism is unable to utilize lactose rapidly or not at all, the slant (where aerobic metabolism has occurred) undergoes a pH change. Neutral end products from the complete breakdown of glucose have replaced the organic acids. The organism, requiring further nutrients, breaks down the peptones in the medium. The alkaline end products from protein metabolism cause the slant, due to the phenol red indicator, to turn red. The anaerobic utilization of glucose in the butt has produced different end products, the most significant of which are stable organic acids. The butt remains yellow throughout the incubation period. TSI which exhibits a red slant over a yellow butt, written as alkaline/acid, has been inoculated with an organism capable of fermenting only glucose. Gas production in the form of CO_2 and/or H_2 may occur when glucose has been fermented. Its presence is indicated by bubbles in the medium, splitting of the medium, or an actual lifting of the medium from the bottom of the tube. The medium does not always trap gas as it is formed and minute amounts may be invisible since no apparent disruption occurred. In instances where gas production is not evidenced, a glucose fermentation test employing either semisolid agar or a Durham insert is performed to confirm the ability or inability of an organism to produce gas from glucose. Gas production is signified by placing the subscript "g" after the term describing the butt pH.

When glucose is utilized and H_2S is produced the TSI pattern appears as a red slant over a black butt. Usually the butt is totally involved, but at times the black color may extend to the lower portion of the slant. The reason the black color is only in the butt is that H_2S production takes place more rapidly in an anaerobic environment. The black color will also usually mask the yellow color indicating an acid pH. This pattern is recorded as Alkaline/Acid H_2S. If gas production is evident this is indicated by a subscript "g" after the terms describing the butt pH and H_2S production.

When both glucose and lactose are fermented the slant and butt, which are yellow during degradation of glucose, remain yellow after depletion of glucose because the organism begins to immediately break down lactose. The 1.0 percent concentration of lactose is sufficient for the 18 to 24 hour period. Since acid products are present, both the slant and butt will be yellow at the conclusion of the incubation period. This TSI pattern is written Acid/Acid. If gas is produced this is indicated by a subscript "g" after the term describing the butt pH. Certain enteric organisms utilize the glucose and lactose so rapidly that the entire slant does not appear acid (yellow) when the reaction is being interpreted. This circumstance, known as "reversion", has occurred because the organism, after depleting both carbohydrates, is in the process of degrading peptones at the termination of incubation. The upper one-half to two-thirds of the slant is red because alkaline end products of protein metabolism have caused the phenol red indicator to revert from yellow to red. The remainder of the slant and the entire butt appear yellow. A true Alkaline/Acid TSI pattern evidences a sharp line of red over yellow at the interface of the slant and butt. Reversion is indicated by writing the pattern Acid /Acid. Usually gas is produced and is indicated by a subscript "g" after the term describing the butt. When both glucose and lactose are utilized and H_2S is produced, the TSI pattern appears as a yellow slant over a black butt. The black color of the butt usually masks the yellow color indicating the acid pH of the butt. This pattern is written Acid/Acid, H_2S. If gas is produced this is indicated by a subscript "g" after the term describing the butt. It is produced by lactose fermenters.

When both sucrose and glucose have been utilized and H_2S is produced the TSI pattern appears as a yellow slant over a black butt. The black color of the butt usually masks the yellow color indicating the acid pH of the butt. This pattern is recorded Acid/Acid H_2S. If gas production is evident this is recorded by a subscript "g" after the terms describing the butt. It is produced by nonlactose fermenters.

Non-enteric gram-negative bacilli which have been inoculated on TSI do not ferment either glucose, sucrose, or lactose. The TSI pattern will appear as a red slant (due to protein metabolism) over a "no change" butt (indicating no growth). This pattern is recorded as Alkaline/No Change.

The primary reason for inoculating enteric bacteria on TSI is to group them according to certain characteristic reactions. *Escherichia coli*, *Klebsiella* species, and *Enterobacter* species exhibit a TSI pattern of Acid/Acid_g. The latter two organisms exhibit reversion of the slant. Organisms which produce a TSI pattern of Alkaline/Acid include *Shigella* species, 15 percent of the *Providencia* species and certain *Proteus* species. The characteristic TSI pattern of other *Proteus* species, and most *Providencia* species is Alkaline/Acid_g. *Arizona* species, *Proteus mirabilis*, *Citrobacter* species, and 98 percent of the *Salmonella* species exhibit an Alkaline/Acid, H_2S, g reaction. *Citrobacter freundii* is a lactose fermenting organism which exhibits an Acid/Acid H_2S pattern. *Proteus vulgaris* is a sucrose fermenting organism which gives an Acid/Acid H_2S reaction. *Pseudomonas* species and *Alcaligenes* are nonfermenting gram-negative bacilli which exhibit an Alkaline/No Change TSI pattern.

This videotape shows a properly prepared TSI tube. The method of inoculation from enteric media will be shown. Intepretation of the TSI patterns is explained. Proper recording of these patterns is stressed.

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This past hour we have discussed the classification, description, and inoculation of TSI. The ingredients which make TSI a differential media have been presented. The method of interpreting each different biochemical reaction has been discussed and shown in the videotape. The interpretation of each of the TSI patterns has been explained and demonstrated on the videotape.

Use and correct interpretation of TSI will enable you to begin accurate identification procedures on enteric bacteria.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
MICROBIOLOGY BRANCH

*LP 08-280-425
058

Kirby Bauer Susceptibility Testing (1 hour)

COURSES PRESENTED TO: 311-92B10 Medical Laboratory Specialist (Basic)

PLACE: Classroom

REFERENCES: GR 09-280-465-1
Bailey, W. R. and Scott, E. G. Diagnostic Microbiology, 4th ed. St. Louis: C. V. Mosby Co., 1974, chap 34.

Lennette, E. H. and others. Manual of Clinical Microbiology. 2nd ed. Washington: American Society for Microbiology, 1974, chap 44 & 46.

STUDY ASSIGNMENT: Not applicable

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: GR 09-280-465-1

PERSONNEL: One Primary Instructor (MOS 92B30)

INSTRUCTIONAL AIDS: Chalk, magnetic chalkboard, and eraser; VT No. 163 and TV receivers (see Annex A)

TROOP REQUIREMENTS: Not applicable

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

I. INTRODUCTION (5 min)

A. Opening statement: While isolation and identification of bacteria are major goals of the clinical bacteriology laboratory, the ultimate objective is the destruction by therapeutic agents of microorganisms responsible for the disease process. As laboratory specialists you will test antibiotics and other chemotherapeutic agents *in vitro* to determine which may be effective against pathogenic microorganisms. Consequently, you will have further aided the physician in his efforts to treat infections.

B. Objectives:

1. Given a list of media, select the one to be used for the Kirby Bauer susceptibility test.

This lesson plan supersedes LP 08-280-425, 026

2. Given a list of procedures, select the steps in preparing inoculum for a Kirby Bauer susceptibility test.

3. Given a list of procedures, select the steps in preparing and inoculating sensitivity plates for the Kirby Bauer susceptibility test.

4. Given a list of possible ways to interpret zone sizes, select the correct method.

C. Class Procedure and Lesson Tie-in: The information from this lecture will be used in a practical exercise designed to acquaint you with preparing, reading, and reporting results of the Kirby Bauer susceptibility test.

II. EXPLANATION (30 min)

A. Purpose:

1. To determine the susceptibility or resistance of an organism to a variety of antibiotics and chemotherapeutic agents

2. Guides the physician's selection of drugs to be used in the treatment of patients.

NOTE: Refer to GR 09-280-465-1 page D-1 thru D-5

B. Medium

1. Mueller-Hinton agar (150 mm plates)

2. 5 percent defibrinated sheep blood may be added to test fastidious organisms

C. Preparation of Inoculum

1. Select 3 to 5 identical colonies

2. Transfer to 3-5 ml of broth media

   a. Mueller-Hinton broth

   b. Trypticase soy broth

3. Incubate 2 to 8 hours at 37 C

4. Compare turbidity to barium chloride standard

   a. If the inoculum is more turbid than the standard, dilute it with sterile normal saline

   b. If the inoculum is less turbid than the standard, incubate it until the desired turbidity is obtained

D. Inoculation and Preparation of Sensitivity Plates

1. Dip sterile cotton swab into inoculum
2. Remove excess inoculum
3. Streak the plate in three different directions with the swab
4. Let the plate dry 3 to 5 minutes
5. Place the antibiotic impregnated disks on the plate
   - a. Types of discs dependent upon
     - (1) Gram reaction of organism
     - (2) Specimen source
     - (3) Physician's preferences
     - (4) Drug availability
   - b. Maximum number of discs dependent upon plate size
     - (1) 15 X 150 mm: 12 discs per plate
     - (2) 15 X 100 mm: 4 discs per plate
6. Press discs lightly to medium with flamed forceps
7. Incubate 18-24 hours at 37 C, regular oxygen tension

QUESTION: Why is increased CO_2 not used?

ANSWER: Because surface pH changes may affect the results. (May significantly alter the zone size.)

E. Interpretation

1. Read the zone size from the reverse side of the plate
2. Observe for zone of inhibition around disc
3. Zone size is determined by

a. Rate of diffusion of the antibiotic

b. Degree of susceptibility of the organism to the antibiotic

4. Measurement of zones of inhibition

a. Template overlay

(1) Innermost circle centered over the disc

(2) Outermost circle shows the minimum zone size to be considered susceptible

b. Ruler or calipers

(1) Measure from endpoint of zone of inhibition to endpoint, through the center of the disc

(2) Compare results to standardized, printed charts to determine susceptibility or resistance

NOTE: A zone which may be susceptible for one antibiotic may be resistant for another.

F. Reporting

1. Susceptible--a zone of inhibition that is greater than the larger size on the reference chart

2. Resistant--a zone of inhibition that is less than the smaller size on the reference chart

3. Intermediate

a. A zone size that is between the limits of susceptible and resistant

b. Usually used only for highly resistant organisms

NOTE: Normally any zone size less than susceptible is reported as resistant

QUESTION: Why is it necessary to measure the zone of inhibition?

ANSWER: The size of the zone is related to the effectiveness of the agent.

G. Videotape- (see Annex A)

1. Introduction

a. Antibiotic susceptibility testing (14.43 min)

b. Points to be observed

   (1) Selection of the organism to be tested

   (2) Preparation of standardized inoculum

   (3) Medium of choice - Mueller-Hinton

   (4) Method of inoculation

   (5) Disc placement and arrangement

   (6) Reading and interpretation of the zone of inhibition

2. Show videotape (TV No. 163)

H. Questions From Students

QUESTION: What is barium sulfate used for in the Kirby-Bauer method?

ANSWER: Barium sulfate is the dilution standard used in the preparation of a standardized inoculum.

III. SUMMARY (5 min)

A. Review of Main Points

1. Purpose
2. Medium
3. Preparation of inoculum
4. Inoculation of plates
5. Interpretation
6. Reporting

B. Closing Statement: The simple, rapid, and accurate Kirby-Bauer single disc method of susceptibility testing is one of the most important procedures you will perform in the clinical laboratory. The usefulness of this procedure is dramatically demonstrated by the response of a patient receiving appropriate therapy for an infectious disease.

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MICROBIOLOGY BRANCH

*IM 08-280-425
058

Antibiotic and Chemotherapeutic Agents

While isolation and identification of bacteria are major goals of the clinical laboratory, the ultimate objective is the destruction by therapy of microorganisms responsible for the disease process. As laboratory specialists you will test antibiotics and other chemotherapeutic agents in vitro to determine which are effective against pathogenic microorganisms. Consequently, you will have further aided the physician in his efforts to treat infections.

Antibiotics are metabolic compounds produced by microorganisms; these compounds are either bactericidal or bacteriostatic against other microorganisms. As with chemical disinfectants and antiseptics, some antibiotics are toxic to both bacteria and host cells, while others can be used topically but not internally to eradicate infecting microorganisms. Antibiotics which can be used to treat internal or external bacterial infections are termed chemotherapeutic agents. Many of these antibiotics have been structurally identified and have been artificially synthesized. They are still defined as antibiotic since their original source was a microorganism.

A chemotherapeutic agent is any compound which can be used to kill infecting microorganisms without causing any appreciable harm to the host cells. Agents with this characteristic possess "selective toxicity" and are very useful in the treatment of systemic infection. "Selective toxicity" is a trait of certain antibiotics, such as pencillin, tetracyclines, and other artificially-synthesized organic compounds, such as sulfonamides and halogenated pyrimidines (5-iododesoxyuridine). The systemic use of these chemotherapeutic agents for treatment of infectious disease is termed chemotherapy.

Antibiotic and chemotherapeutic susceptibility testing is a qualitative procedure to determine which pathogenic organisms are susceptible or resistant to various antibiotics and chemotherapeutic agents. The method currently in use is the Kirby-Bauer single disc method of susceptibility testing. This method is a highly standardized and accurate procedure which utilizes an antibiotic impregnated disc on Mueller-Hinton agar.

The videotape demonstrates the procedures involved in performing this test. The selection of a pure culture for the inoculum is stressed. The preparation of a standardized inoculum by the use of a 1 percent barium sulfate optical standard is shown. A sterile cotton swab is saturated with the inoculum and the medium is streaked horizontally, vertically, and double diagonally to insure complete seeding. The medium of choice for the Kirby-Bauer method is Mueller-Hinton agar, with or without supplements.

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*This instructor's manuscript supersedes IM 08-280-425, 026

This is poured in large (15 cm) plates so that an entire zone of inhibition can be measured. Disc selection, placement, and arrangement are all shown very well. Firm placement of the discs is important to insure proper diffusion of the chemotherapeutic agent through the medium. Proper arrangement of the discs is necessary for a complete zone of inhibition. Measurement of the zone of inhibition with calipers and other instruments is demonstrated. Comparison of the measured zone to the commercial chart is shown. Interpretation of the results to include susceptible, intermediate, and resistant is given in some detail.

Although optochin and bacitracin are not truly antibiotics nor chemotherapeutic agents, the methods for performing susceptibility tests with these compounds is similar to antibiotic and chemotherapeutic susceptibility testing. Results of these susceptibility tests will identify certain organisms in the genus Streptococcus. The procedure for performing these tests is outlined in the videotape. A pure culture of the organism to be tested is streaked onto a sheep blood agar plate in vertical, horizontal, and double diagonal directions. The discs impregnated with optochin or bactracin are placed on the medium, which has been streaked with forceps which have been alcohol flamed. The disc is gently tapped with the forceps to insure firm contact is made with the medium. Plates are now incubated and observed for zones of inhibited growth. When a zone of inhibition is observed, the organism is reported as being susceptible. If no zone of inhibition is present, the organism is reported as being resistant. Before the results of the test are valid, three criteria must be met: there must be a pure culture, good growth, and confluent growth of the organism being tested.

During this hour of instruction we have defined two terms associated with antibiotic susceptibility testing. The first of these, antibiotic, is a product of living microorganisms which is bactericidal or bacteriostatic against other microorganisms. The second, chemotherapeutic agent, exhibits "selective toxicity." We have also demonstrated and discussed the Kirby-Bauer single disc method of susceptibility testing.

The simple, rapid, and accurate Kirby-Bauer single disc method of susceptibility testing is one of the most important tools available to you in the clinical bacteriology laboratory. The usefulness of this procedure is dramatically demonstrated by the response of a patient receiving appropriate therapy for an infectious disease.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-426
LABORATORY SCIENCES DIVISION 048
MICROBIOLOGY BRANCH

Enteric Biochemicals - 2 hours

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES:

GR 09-280-465-1
Baltimore Biological Laboratories. BBL Manual of Products and Laboratory Procedures. 5th ed. Cookeysville, BBL Division of Bio Quest, Division of Becton, Dickinson, and company, 1973.
Difco Laboratories, Inc. Difco Manual of Dehydrated Culture Media and Reagents for Microbiological and Clinical Laboratory Procedures. 9th ed. Detroit: Difco Laboratories, Inc. 1971.

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: M 08-280-426-1, issued prior to class

PERSONNEL: One instructor (MOS 92B30)

INSTRUCTIONAL AIDS: Chalk, magnetic chalkboard, and eraser; MP 08-280-426-1 thru 18 (see Annex A)

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

I. INTRODUCTION (5 min)

A. Opening Statement: Although you have discovered that the various species of enteric bacteria resemble one another morphologically and have certain biochemical characteristics in common, experience with the use of TSI has indicated to you that these organisms can be differentiated by biochemical means. In the laboratory you will employ a series of biochemical tests to identify the various genera of enteric bacteria.

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*This lesson plan supersedes LP 08-280-426, 036

B. Objectives:

1. Given a list of enteric biochemicals, select the correct method of inoculation and incubation.

2. Given a description of properly inoculated and incubated enteric biochemicals, select the correct reporting terminology for those biochemicals.

3. Given an enteric biochemical, select the biochemical test principal involved in its use.

C. Class Procedure and Lesson Tie-in: A practical exercise in inoculating, interpreting, and reporting of biochemicals will be conducted during the accompanying PE.

II. EXPLANATION: (70 min)

A. General Considerations

1. Used in various combinations (batteries) to identify enteric and the related gram negative bacilli

NOTE: The types of biochemicals utilized in a battery are dependent upon the TSI reactions and the specimen source.

2. Must be inoculated with a pure culture

   a. Inoculate from a TSI tube

   b. Inoculate with a sterile inoculating needle

3. Inubation--18 to 24 hours at 37 C

B. Types of Tests

1. Phenylalanine deaminase test

   a. Principle: to determine the ability of an organism to deaminate phenylalanine to phenyl pyruvic acid which, with the ferric ion in the reagent, forms a green color

   b. Type of Medium--solid agar slant

   c. Inoculation--streak slant

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d. Interpretation

(1) Uninoculated--white slant

(2) Reagent

(a) 10% ferric chloride $FeCl_3$

(b) Place 2 to 3 drops on the slant

(c) Add after incubation and before interpretation

(3) Positive--green color on slant

(4) Negative--no green color (the ferric chloride may give the slant a yellow color)

e. Precautions

(1) Color reaction fades after several minutes

(2) Green color considered positive only after addition of reagent

NOTE: Members of the genus Pseudomonas can produce a blue-green pigment which would be visible before the addition of the $FeCl_3$.

QUESTION: What color is a positive phenylalanine deaminase test?

ANSWER: Green color on the slant.

2. Indole

a. Principle: To determine the ability of an organism to split indole from the tryptophane molecule. Indole reacts with the reagent to form a red color in the reagent.

b. Type of medium--broth

c. Inoculation--tilt tube method

d. Interpretation

(1) Uninoculated--Clear, Yellow-white

(2) Reagent

(a) Kovac's reagent

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(b) Add 3 to 5 drops to the test

(c) Add after inubation and before interpretation

(3) Positive--red reagent ring on surface of medium

(4) Negative--yellow reagent ring on surface of medium

e. Precautions--refraction of light through meniscus of broth may appear red. Do not confuse this color with the reagent layer.

3. Motility with TTC

a. Principle: to determine the ability of an organism to demonstrate movement in a semisolid medium. Enterics reduce TTC, producing a red color where organisms are growing

b. Type of Medium--semisolid agar

c. Inoculation--stab medium one half of its depth

d. Interpretation

(1) Uninoculated--cloudy white (no slant)

(2) Positive--red color diffusing into medium from stab line

(3) Negative--red color concentrated only along stab line (no color diffusion)

e. Precautions

(1) Too much inoculum can produce a heavy amount of red color and can mask a positive or appear falsely positive. Therefore, stab the medium only one time making sure not to disturb the medium outside of the stab line area.

(2) Stabbing too far into the medium can cause false positive interpretation.

QUESTION: What is the reduced color of TTC?

ANSWER: Red.

4. Urease

   a. Principle: to determine the presence of the urease enzyme which hydrolyzes urea. Hydrolysis results in an alkaline pH which turns the pH indicator phenol red to a red color.

   b. Type of medium--broth

   c. Inoculation--tilt tube method

   d. Interpretation

      (1) Uninoculated--yellow-orange or straw

      (2) Positive--pink to red

      (3) Negative--yellow-orange or straw (no color change)

   e. Precautions

      (1) A light inoculum will yield a weak color reaction

      (2) Compare apparent negatives to uninoculated tube to detect weak positive reactions.

5. ONPG (O NitroPhenyl-beta-Galactopyranoside)

   a. Principle: to determine the presence of beta-galactosidase and permease enzymes which break down O-nitrophenyl-beta-galactopyranoside to O-nitrophenyl which is yellow in color.

   b. Type of medium--sterile saline with ONPG impregnated disk

   c. Inoculation

      (1) Agitate needle in saline to produce a heavy suspension of bacteria (visibly turbid)

      (2) Add the ONPG disk (before incubation)

   d. Interpretation

      (1) Uninoculated--clear saline

      (2) Reagent

         (a) ONPG disk

         (b) Added after inoculation and before incubation

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(3) Positive--any yellow color

(4) Negative--no yellow color (colorless)

e. Precautions

(1) Saline must be turbid for clear cut color reactions

(2) Do not misread turbidity as being faintly yellow

QUESTION: What color is a positive ONPG?

ANSWER: Yellow.

6. Lysine decarboxylase

a. Principle: to determine the enzymatic ability of an organism to decarboxylate the amino acid lysine and form cadaverine. The resulting alkaline pH gives a purple color to the pH indicator brom cresol purple.

b. Type of medium--broth

c. Inoculation

(1) Tilt tube method

(2) Inoculate two tubes: one test and one control (the control tube does not have the amino acid in it)

(3) After inoculation overlay both tubes with approximately 0.5 ml of sterile mineral oil

d. Interpretation

(1) Uninoculated--clear purple

(2) Positive--cloudy purple (not obviously yellow)

(3) Negative

(a) Turbid yellow

(b) Clear purple

e. Precautions

(1) Test results cannot be interpreted unless the control tube is yellow

(2) If one tube is turbid then both tubes must be turbid for test results to be considered valid.

QUESTION: What color change indicates a negative lysine decarboxylase test?

ANSWER: Yellow.

QUESTION: What do the lysine decarboxylase, phenylalanine deaminase, and the urease tests have in common?

ANSWER: They all are tests used to determine the presence of a particular enxyme system in a bacteria.

III. SUMMARY (5 min)

A. Review of Main Points

1. General considerations

2. Types of tests

B. Closing Statement: The proper inoculation, incubation, and interpretation of the enteric biochemical tests will allow the laboratory specialist to identify various enteric bacteria. The rapid identification of enteric bacteria will ensure quality patient care and assist in the recovery of debilitated personnel.

ANNEX A
INSTRUCTIONAL AIDS

Magnetic Placards

MP 08-280-426-1	ENTERIC BIOCHEMICALS
MP 08-280-426-2	GENERAL CONSIDERATIONS
MP 08-280-426-3	TYPES OF TESTS
MP 08-280-426-4	PHENYLALANINE DEAMINASE
MP 08-280-426-5	PRINCIPLE
MP 08-280-426-6	INOCULATION
MP 08-280-426-7	INTERPRETATION
MP 08-280-426-8	REAGENT
MP 08-280-426-9	UNINOCULATED
MP 08-280-426-10	NEGATIVE
MP 08-280-426-11	POSITIVE
MP 08-280-426-12	LYSINE DECARBOXYLASE
MP 08-280-426-13	UREASE
MP 08-280-426-14	TYPE OF MEDIUM
MP 08-280-426-15	INDOLE
MP 08-280-426-16	PRECAUTIONS
MP 08-280-426-17	MOTILITY WITH TTC
MP 08-280-426-18	O-NITROPHENYL-BETA-GALCTOPYRANOSIDE (ONPG)

NOTE: These placards are used as main headings throughout part II.

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MODIFICATIONS

LP# 08-280 428 of this publication has (have) been deleted in adapting this material for use in Vocational and Technical Education. Deleted material involves extensive use of military forms, procedures, systems, etc. and was not considered appropriate for use in vocational and technical education.

SSG A. Mc Math 1112

ACADEMY OF HAEALTH SCIENCES, UNITED STATES ARMY LP 08-280-429
LABORATORY SCIENCES DIVISION 048
MICROBIOLOGY BRANCH

The Bacterial Stool Specimen 1 hour

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist, Basic

PLACE: Classroom

REFERENCES:
Bailey, W. R., and Scott, E. G. Diagnostic Microbiology. 4th ed. St. Louis; C. V. Mosby Company, 1974, chapter 9
Lennette, E. H., and others (ed). Manual of Clinical Microbiology. 2nd. ed. Washington: American Society for Microbiology, 1974 chap 6.

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT AND MATERIALS: None

PERSONNEL: One instructor MOS 92B3H

INSTRUCTIONAL AIDS: Chalk, chalkboard, and eraser

TROOP REQUIREMENTS: None

TRANSPORTAION REQUIREMENTS: None

METHOD OF INSTRUCTION: Lecture

I. INTRODUCTION: (5 min)

A. Opening Statement: Approximately fifty percent of a normal stool specimen is composed of bacteria. This high concentration of microorganisms is a normal situation. A medical laboratory technician working in a Bacteriology Department will be required to evaluate this high bacterial flora and isolate from the many bacteria present those that may cause a pathologic condition.

B. Objectives:

1. From a list of bacteria, select five that are intestinal pathogens

2. Given a list of various media, select those to be inoculated for a stool culture.

3. Given a situation, select the steps and/or procedures used to [illegible] pathogens.

This LP Supersedes LP 08-280-429, [illegible]

C. Class Procedure and Lesson Tie-in: The information from this lecture, in conjunction with other lectures relate to the enterics, and will be used in an upcoming stool specimen PE. Stool specimens will also be included in the final crucial PE.

II. *EXPLANATION:* (30 min)

A. Intestinal Pathogens

1. *Salmonella* species

a. Typhoid fever

b. Food poisoning

2. Shigella species--bacillary dysentary

3. Vibrio species--cholera

4. Enteropathogenic *Escherichia* *coli*--infantile diarrhea

5. *Arizona* species--gastroenteritis

B. Other Organisms Capable of Causing Disease in the Digestive Tract

1. *Staphylococcus* *aureus*--food poisoning

2. *Clostridium* species

a. Food poisoning

b. Botulism

3. These organisms are pathogenic by means of their toxins being ingested by man

4. They are usually identified from food cultures at the doctor's request

C. Types of Specimens Submitted to the Laboratory

1. Rectal swab

2. Fecal material

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D. Media used for Inoculation of the Stool Specimen

1. Most commonly used media

   a. Hektoen agar

   b. MacConkey agar

   c. GN Hajna broth

2. Other media often used and organisms commonly isolated

   a. Eosin methylene blue agar (EMB)--*Escherichia coli*

   b. XLD agar (xylose lysine desoxycholate agar)

      (1) *Salmonella* Species

      (2) *Shigella* species

   c. Mannitol salt agar--*Staphylococcus aureus*

   d. Sheep blood agar

      (1) *Staphylococcus aureus*

      (2) Enteropathogenic *Escherichia coli*

   e. TCBS agar (thiosulfate citrate bile salts sucrose)-*Vibrio* species

E. Processing of the Specimen

1. Inoculate specified media and incubate

   a. Plate media: 18-24 hours

   b. GN Hajna: 6-8 hours

2. Subculture GN Hajna

   a. To specified plate media

   b. Incubate plates 18-24 hours

3. Screen for possible pathogens
    a. MacConkey agar
        (1) Non-lactose fermenters
        (2) Also rapid lactose fermenters on infants
    b. Hektoen--blue-green colonies
4. Inoculate possible pathogens to TSI and incubate
5. Inoculate biochemicals as prescribed by the laboratory SOP
6. Preliminary report sent out on presumptive pathogens
7. Confirm identification of enteric pathogens by serological testing
8. Perform antibiotic susceptibility tests on all pathogens
9. Final report sent to doctor/clinic as soon as possible

III. SUMMARY (5 min)

A. Review of Main Points
    1. Intestinal pathogens
    2. Media
    3. Processing

B. Closing Statement: Proper handling and processing of stool specimens is necessary for efficiently returning results to the doctor or clinic with speed, accuracy, and economy.

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SP5 Mary Jo Garis
*LP 08-280-431
058

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
MICROBIOLOGY BRANCH

Aerobic/Anaerobic Practical Exercise (2 hours)

COURSES PRESENTED TO: 311-92B10, Basic Medical Laboratory Specialist course

PLACE: Classroom

REFERENCES: All previous references

STUDY ASSIGNMENT: Before class read M 08-280-431-1

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: See Annexes A and B

PERSONNEL: Eight instructors (MOS 92B4H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, and eraser

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Practical Exercise

I. INTRODUCTION (5 min)

A. Opening Statement: During this exercise, the remainder of this course, and after your assignment to clinical laboratories, you will be required to work with bacteria isolated from many different sources. This exercise is designed to give you practice in performing those procedures routinely used for isolating bacteria.

B. Objective

Given known bacterial specimens, inoculate and streak media for isolation; observe and interpret the oxygen requirements of each of the given bacteria, as demonstrated in class.

C. Class Procedure and Lesson Tie-In

1. Application of information received during previous lectures and demonstrations.

2. Familiarization with culture techniques of anaerobic bacteria, conducted in two hours on two consecutive days, one hour each day. Specific tasks assigned by instructor during each phase will lead to completion of exercise by final hour.

---

*This Lesson Plan supersedes LP 08-280-431, 046

II. APPLICATION (70 min)

A. Instructors' Activities

1. Class divided into two-student teams.

2. Equipment and materials issued (see Annexes A and B).

3. Pure cultures prepared and issued (see Annex C, day prior to PE)

4. Daily instructions given in step-by-step fashion.

5. Previous day's activities reviewed.

6. Questions answered.

7. Corrections made.

8. Laboratory safety maintained.

9. Exercise classroom control.

B. Students' Activities

1. Aseptic technique maintained.

2. Appropriate media inoculated and streaked for isolation; placed in appropriate oxygen environment (see M 08-280-431-1).

3. Plates observed for growth (see M 08-280-431-1).

4. Culture materials discarded.

5. Clean up as directed.

6. Equipment stored properly.

C. Organization of Instructional Hours

1. First day (40 min) (see Annexes A, B, C)

a. Selection of cultures by students

NOTE: Each group of two students, receives four organisms divided into two groups of two each, labeled A and B. Each member of every group will receive two organisms. All different oxygen requirements will be represented.

b. Cultures inoculated and streaked (see M 08-280-431-1)

c. Cultures incubated

d. Specimens properly discarded

e. Clean up performed

2. Second day (30 min) (see Annexes A and B)

a. Cultures removed from incubator

b. Record observations

c. All culture materials properly discarded

d. Clean up performed

III. REVIEW (10 min)

A. Questions from students

B. Review of Main Points

1. Collection of anaerobic specimens

2. "Challenge" plates

3. Use and preparation of media

C. Closing Statement: Background knowledge of the theories and principles of clinical bacteriology is insufficient. You must develope the ability to apply that knowledge in an organized manner so that you can better perform your duties as a clinical bacteriology technician.

Tools, Equipment, and Materials

1.	Autoclave	1 per class
2.	Incubator	1 per class
3.	Loop, inoculating	1 per student
4.	Needle, inoculating	1 per student
5.	Marker, wax	1 per student
6.	Disinfectant, germicidal	2 gallons per class
7.	Detergent, surgical	1 gallon per class
8.	Burner, gas, touchmatic	1 per student
9.	Bucket, metal, decontamination	4 per class
10.	Towels, paper	7 pks per class
11.	Tape, masking	3 rolls per class
12.	Canister, metal, plate	1 per student
13.	Jar, anaerobic, complete	10 per class
14.	Gas pack, anaerobic, with indicator	1 per anaerobic jar
15.	Swabs, sterile	250 tubes per class
16.	Racks, test tube, 40 hole	20 per class
17.	Syringe, 10cc	10 per class

ANNEX B

Media and Reagents

1. Sheep Blood Agar (SBA)	4 per student
2. Thio 135C with supplements	2 tubes per student
3. Todd-Hewitt broth (50 ml/bottle	3 bottles
4. Blood culture bottle	1 bottle
5. Sterile needle and syringe	1 per class

ANNEX C

Procedure for Preparation of Practical Exercise

A. Materials

1. Three bottles of Todd-Hewitt broth (50 ml/bottle)

2. One bottle of blood culture broth

NOTE: These quantities are based on forty students.

3. Sheep Blood Agar cultures

    a. *Staphylococcus aureus*

    b. *Escherichia coli*

    c. *Pseudomonas* species

    d. *Clostridium* species

4. Forty-hole racks (one per group of two students)

5. Swab tubes (sterile) (two per student)

6. Masking tape (1 roll)

7. Burner with tubing

8. Felt tip marker

9. Sterile swabs and inoculating loop

10. Disinfectant

11. CO_2 incubator

B. Preparation Procedure

1. Day prior to practical exercise

    a. Inoculate Todd-Hewitt broths with organisms from appropriate culture plates, except *Clostridium*, using a sterile loop. Insure that all bottles are labeled appropriately.

    b. Inoculate the blood culture bottle with *Clostridium*.

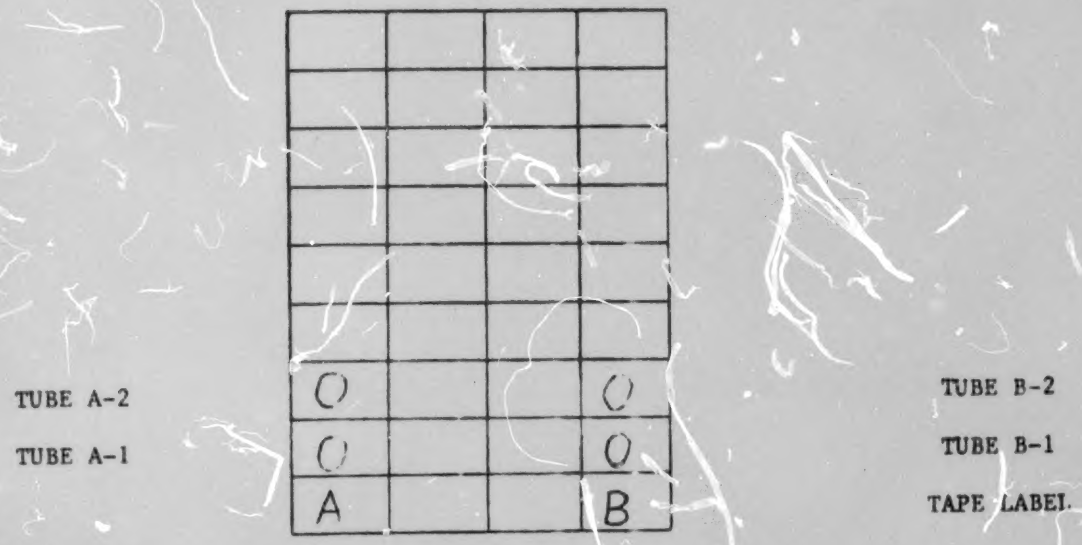
    c. Incubate all inoculated media

2. Day of P.E. - Dip swab tubes into appropriate cultures and distribute in accordance with protocol 1 (one rack of specimens per group of two).

PROTOCOL 1

GROUP A	1.	*Escherichia coli*
	2.	*Pseudomonas*
GROUP B.	1.	*Clostridium*
	2.	*Staphylococcus aureus*

DIAGRAM



NOTE: One rack arranged in this manner is needed for every two students. Care should be taken to arrange the organisms exactly as indicated so that they will match the students instruction sheet (M 08-280-431-1)

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY   M 08-280-431-1
LABORATORY SCIENCE DIVISION   038
MICROBIOLOGY BRANCH

Procedure for Known Aerobe/Anaerobe Practical Exercise

First Day

1. The class will be divided into groups of two students each. Each group will be issued four cultures in one rack as indicated in figure 1 below. Each student in the group should choose either A or B.

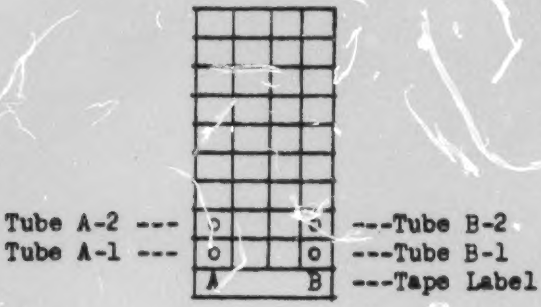


Figure 1

2. Each student will obtain four sheep blood agar (SBA) plates and two thio 135 C tubes. Label two SBA for each culture on the back of the plate, using a wax marker. One plate should be labeled O_2, and the other labeled ANA, for each specimen. The plate should also be labeled with the specimen number and student number (i.e., A-1 O_2).

3. Using aseptic technique, quickly inoculate the labled media by rolling the swab on a small portion of the surface of the SBA plate. Streak plates for isolation using the four quadrant method. Specimens suspected of containing anaerobes should be handled with care and speed, as oxygen is toxic to some organisms.

4. Using aseptic technique, inoculate only the top portion of a labeled thio 135 C tube with the appropriate specimen.

5. Stack the SBA plates labeled O_2 in a metal canister. Store in the CO_2 incubator at 37 C as directed by an instructor.

6. Place thio 135 C in a 40 hole rack.

7. Place plates labeled ANA in the anaerobic jar. Obtain one gas pack generator and one indicator strip per anaerobic jar. Obtain a syringe, and fill with 10 cc of tap water. Tear off the corner indicated on the gas pack and fill the chamber using the pre-filled syringe. Place the gas pack in the jar. Open the indicator strip to the line indicated on the packet. Place in a jar so indicator can be seen. Place

lid on the jar. Tighten the thumb screw "finger tight".

8. Place both the rack of tubes and the anaerobic jar on the cart to be incubated at 37 C as directed by an instructor.

9. Clean up as directed.

Second Day

1. Remove cultures from incubators.

2. Observe and compare the O_2 plate and the ANA plate for each specimen. Read both yours and your partner's.

3. Observe the thio 135 C tubes for both A and B sets. Note the area of growth in each tube.

4. Consider all characteristics. Are your results consistent with the following key?

Number		Growth Pattern	Conclusion
A-1	O_2	growth	facultative anaerobe
	ANA	growth	
A-2	O_2	growth	strict aerobe
	ANA	no growth	
B-1	O_2	no growth	strict anaerobe
	ANA	growth	
B-2	O_2	growth	facultative anaerobe
	ANA	growth	

5. Compare the thio 135 C tubes with the illustration in figure 2. Are the growth patterns consistent with your conclusions?

Oxygen Requirements of Microorganisms

Organisms are classified according to their oxygen requirements.

1. Aerobes--require normal atmospheric oxygen.
2. Microaerophiles--require reduced atmospheric oxygen.
3. Anaerobes--require atmosphere devoid of free oxygen.
4. Facultative anaerobes--adapt to any oxygen environment.

One may attach the adjectives "obligate" and "facultative" to the above terms to expand their meaning. Obligate means strict or absolute, while facultative means flexible; therefore an obligate anaerobe is a strict or absolute anaerobe and can never be anything else.

Most pathogens are aerobes, but their growth is enhanced in the presence of CO_2. Care must be exercised not to confuse the terms "aerogenic" and "anaerogenic" with "aerobic" and "anaerobic".

THIOGLYCOLLATE BROTH

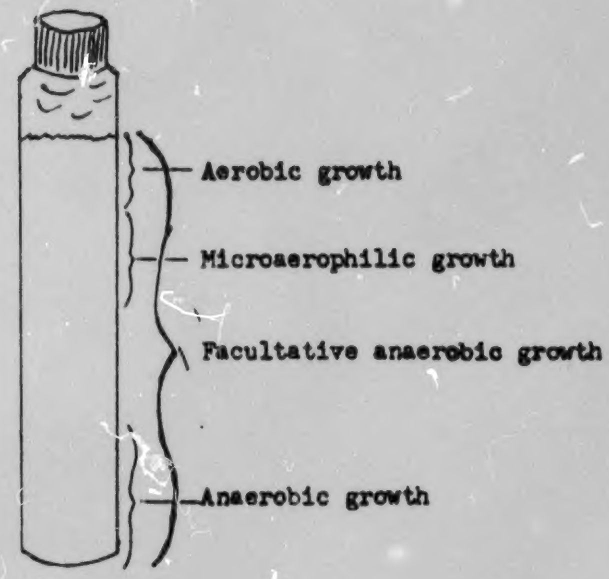


Figure 2

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY M 08-280-432
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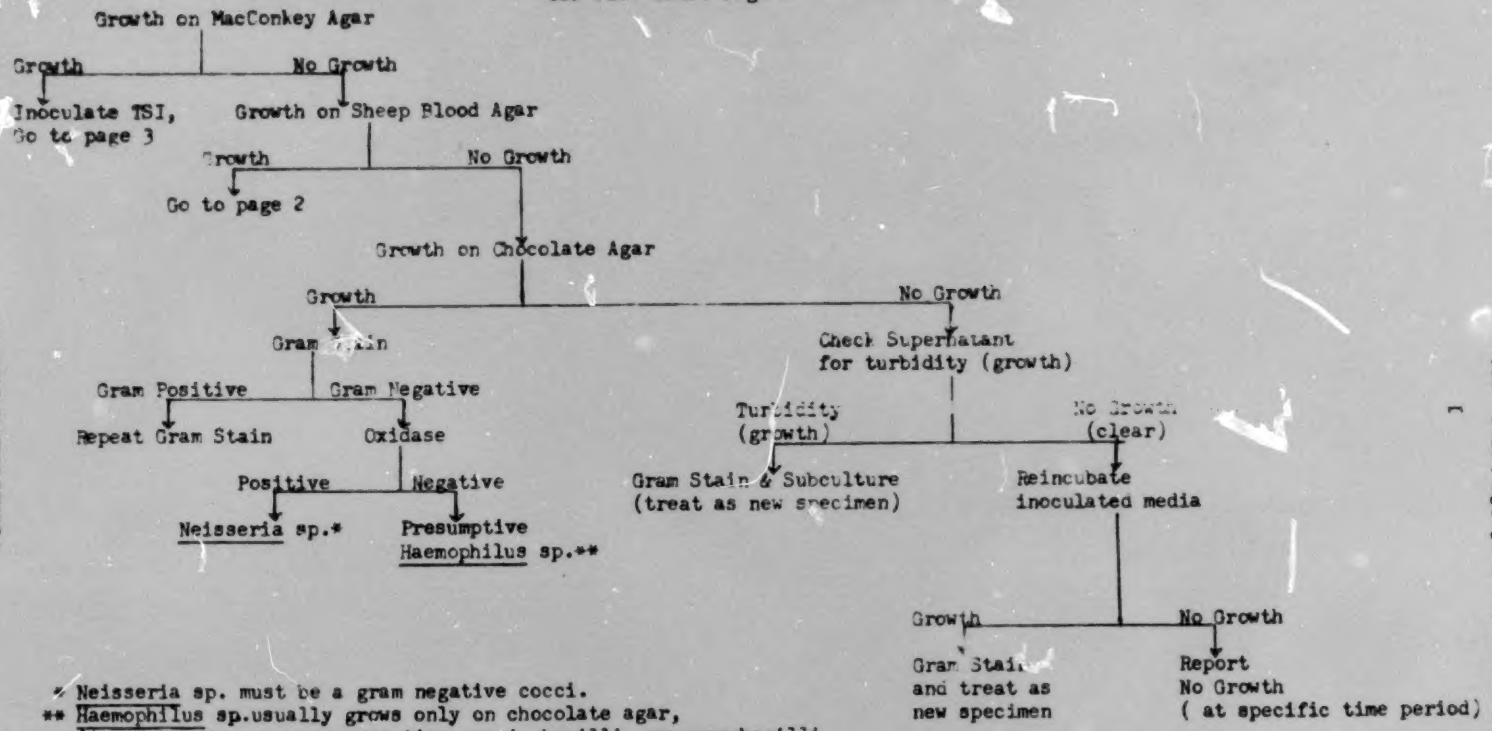
LABORATORY SCIENCES DIVISION
MICROBIOLOGY BRANCH

CSF Flow Chart Page 1

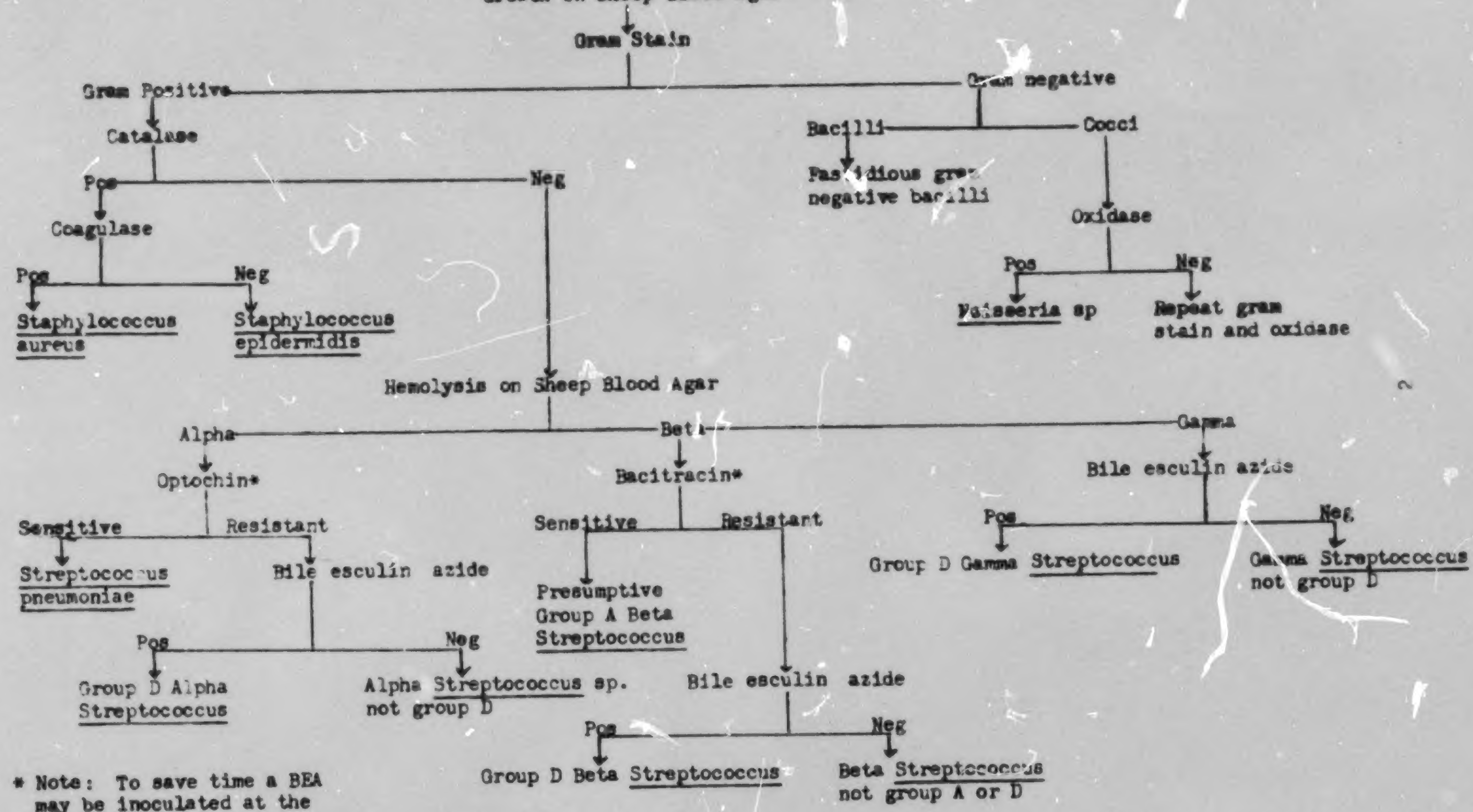

Growth on MacConkey Agar
Growth
No Growth
Inoculate TSI, Go to page 3
Growth on Sheep Blood Agar
Growth
No Growth
Go to page 2
Growth on Chocolate Agar
Growth
No Growth
Gram Stain
Gram Positive
Gram Negative
Repeat Gram Stain
Oxidase
Positive
Negative
Neisseria sp.*
Presumptive Haemophilus sp.**
Check Supernatant for turbidity (growth)
Turbidity (growth)
No Growth (clear)
Gram Stain & Subculture (treat as new specimen)
Reincubate inoculated media
Growth
No Growth
Gram Stain and treat as new specimen
Report No Growth (at specific time period)

* Neisseria sp. must be a gram negative cocci.

** Haemophilus sp. usually grows only on chocolate agar, It may appear as a gram negative cocci, bacilli, or coccobacilli.

This mimeo supersedes M 08-280-432-1, 016

CSF Flow Chart Page 2


Growth on Sheep Blood Agar
Gram Stain
Gram Positive
Gram negative
Catalase
Pos
Neg
Coagulase
Pos
Neg
Staphylococcus aureus
Staphylococcus epidermidis
Bacilli
Cocci
Fastidious gram negative bacilli
Oxidase
Pos
Neg
Neisseria sp
Repeat gram stain and oxidase
Hemolysis on Sheep Blood Agar
Alpha
Beta
Gamma
Optochin*
Sensitive
Resistant
Streptococcus pneumoniae
Bile esculin azide
Pos
Neg
Group D Alpha Streptococcus
Alpha Streptococcus sp. not group D
Bacitracin*
Sensitive
Resistant
Presumptive Group A Beta Streptococcus
Bile esculin azide
Pos
Neg
Group D Beta Streptococcus
Beta Streptococcus not group A or D
Bile esculin azide
Pos
Neg
Group D Gamma Streptococcus
Gamma Streptococcus not group D

* Note: To save time a BEA may be inoculated at the same time susceptibility tests are inoculated.

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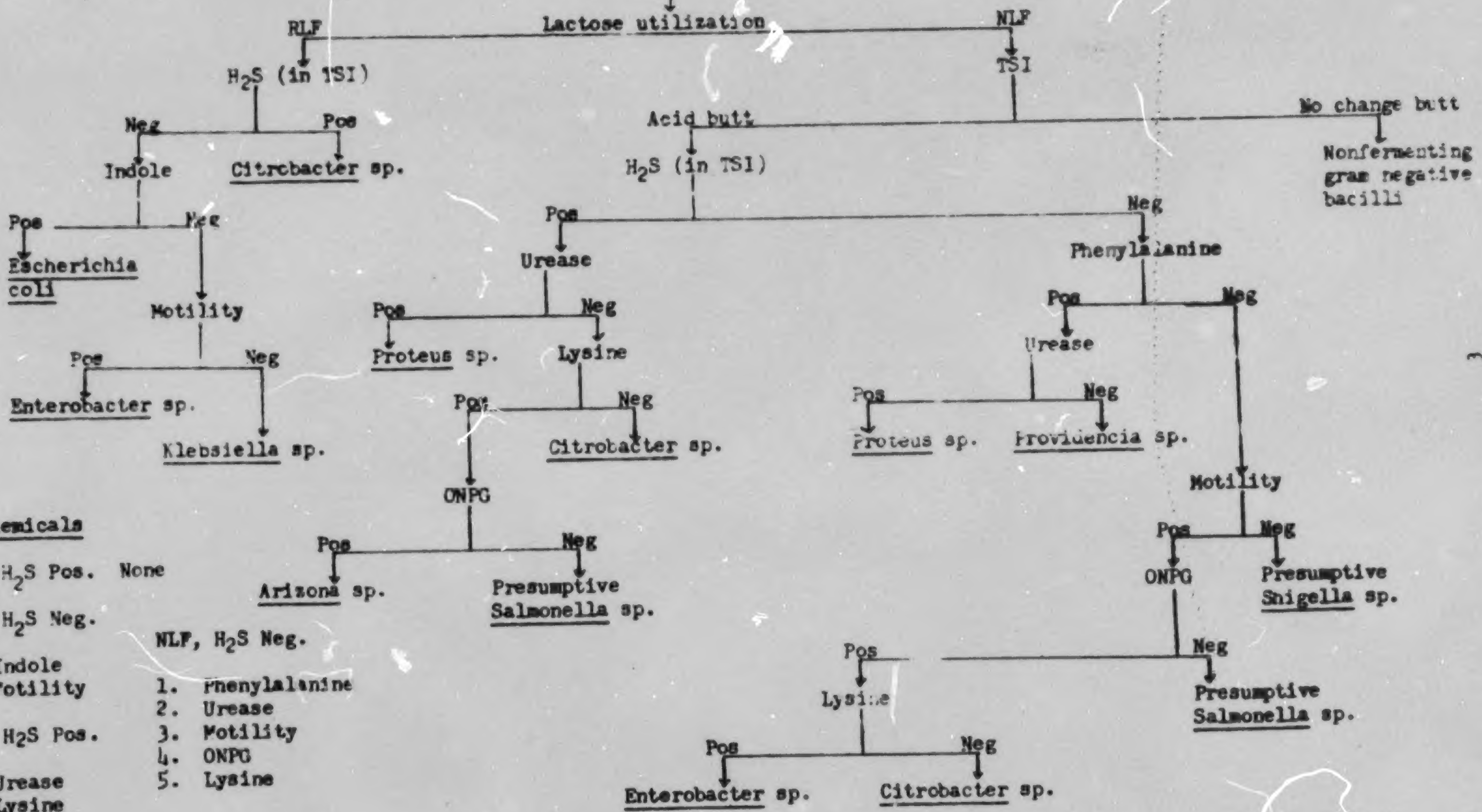
CSF Specimen Flow Chart Page 3
Growth on MacConkey


Lactose utilization
RLF
NLF
H2S (in TSI)
Neg
Pos
Indole
Citrobacter sp.
Pos
Neg
Escherichia coli
Motility
Pos
Neg
Enterobacter sp.
Klebsiella sp.
TSI
Acid butt
No change butt
Nonfermenting gram negative bacilli
H2S (in TSI)
Pos
Neg
Urease
Pos
Neg
Proteus sp.
Lysine
Pos
Neg
Citrobacter sp.
ONPG
Pos
Neg
Arizona sp.
Presumptive Salmonella sp.
Phenylalanine
Pos
Neg
Urease
Pos
Neg
Proteus sp.
Providencia sp.
Motility
Pos
Neg
ONPG
Presumptive Shigella sp.
Pos
Neg
Presumptive Salmonella sp.
Lysine
Pos
Neg
Enterobacter sp.
Citrobacter sp.

Biochemicals

RLF, H_2S Pos. None

RLF, H_2S Neg.

1. Indole
2. Motility

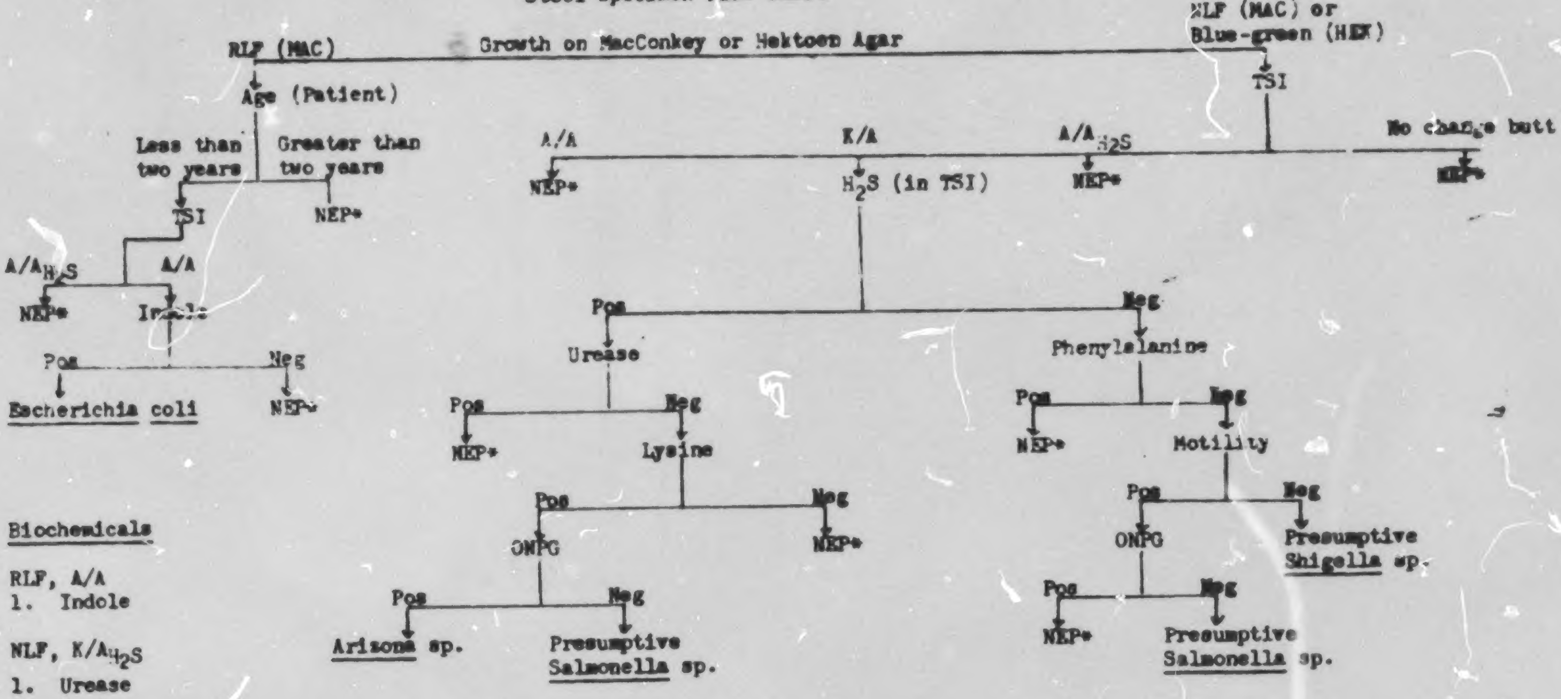
NLF, H_2S Pos.

1. Urease
2. Lysine
3. ONPG

NLF, H_2S Neg.

1. Phenylalanine
2. Urease
3. Motility
4. ONPG
5. Lysine

Stool Specimen Flow Chart


Growth on MacConkey or Hektoen Agar
RLF (MAC)
NLF (MAC) or
Blue-green (HEK)
Age (Patient)
Less than
two years
Greater than
two years
NEP*
TSI
A/A$_{H_2S}$
A/A
NEP*
Indole
Pos
Neg
Escherichia coli
NEP*
TSI
A/A
K/A
A/A$_{H_2S}$
No change butt
NEP*
H_2S (in TSI)
NEP*
NEP*
Pos
Neg
Urease
Phenylalanine
Pos
Neg
NEP*
Lysine
Pos
Neg
NEP*
Motility
Pos
Neg
ONPG
NEP*
Pos
Neg
ONPG
Presumptive
Shigella sp.
Pos
Neg
Arizona sp.
Presumptive
Salmonella sp.
Pos
Neg
NEP*
Presumptive
Salmonella sp.

Biochemicals

RLF, A/A
1. Indole

NLF, K/A$_{H_2S}$
1. Urease
2. Lysine
3. ONPG

NLF, K/A
1. Phenylalanine
2. Motility
3. ONPG

*NOTE: "NEP" Stands for "Not an enteric pathogen"

M 08-280-432
028

SSG Philip A. Salerno

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-433
LABORATORY SCIENCES DIVISION 048
MICROBIOLOGY BRANCH

Mixed Organism - Practical Exercise (12 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES: All appropriate references

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: See Annexes A through D. Refer to M 08-280-433-1, M 08-280-433-2.

PERSONNEL: Eight (8) instructors (MOS 92B3H)

INSTRUCTIONAL AIDS: Chalk, magnetic chalkboard, and eraser; see Annexes A through D

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Practical exercise

I. INTRODUCTION (5 min)

A. Opening Statement: This practical examination is designed to test your working knowledge of the enteric bacteria and the other general pathogens you have studied. Emphasis will be placed on your ability to use proper aseptic technique in all phases of this examination. You will be working with stock cultures. Utilizing all laboratory test procedures appropriate to the specimens, you will isolate and identify each organism present.

B. Objectives

Given necessary materials and simulated throat, stool, and cerebrospinal fluid specimens, select proper media; correctly

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*This lesson plan supersedes LP 08-280-433, 046

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inoculate and streak agar plates; select proper incubation environment; correctly perform appropriate tests and stains; and correctly interpret and report results of tests and identity of bacteria given as demonstrated in class.

C. Class Procedure and Lesson Tie-in

This examination tests the student's comprehension of the identification methods used for general and enteric bacteria.

II. APPLICATION (470 min)

A. Instructors' Activities

1. Insure availability of supplies and equipment (see Annex A).

2. Prepare simulated specimens (see Annexes C and D).

3. Assign specimens to students (see Annex D).

4. Emphasize clinical data.

5. Conduct quality control on all

   a. Media

   b. Reagents

   c. Oragnisms

6. Provide students with the procedure sheet (M 08-280-433-1).

7. Emphasize to students the necessity of complete and legible report sheets (M 08-280-433-2).

8. Advise students that they may use any reference desired but all procedures or schema must agree with M 08-280-432-1

9. Answer students' questions concerning technique and procedure during the examination without providing solutions to identification (answer a question by asking a question).

10. Insure standards of laboratory safety.

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11. Permit students to take break periods in consonance with their speed and ability.

12. Insure daily clean up.

13. Advise students of the method of grading.

14. Observe and record each student's daily results on M 08-280-433-3.

B. Students' Activities

1. Obtain a set of unknown specimens from the instructor.

2. Prepare a worksheet for specimens.

3. Inoculate media suitable for the isolation of unknown organisms.

4. Perform identification procedures appropriate for each organism isolated.

5. Use proper aseptic technique during all procedures.

6. Ask questions concerning overall the examination, but do not ask questions pertaining to test procedures, results, or identification of bacteria.

7. Use all references at your disposal, but all procedures of the schema must agree with M 08-280-432-1.

8. Record all pertinent data in a correct, legible, and neat manner on your work sheet.

9. Turn-in laboratory worksheets at the time designated by the instructor.

10. Restore laboratory to proper order at the end of each day's activities.

C. Organization of Instructional Hours

1. First day (Hours 1-4)

   a. Select appropriate media

b. Centrifuge the two spinal fluid specimens, and along with the three throat and three stool specimens, inoculate and streak all appropriate plate media.

c. Insure that the throat specimen plates are stabbed in all the quadrants.

d. An initial gram stain must be performed on the spinal fluid specimens and turned into the instructors by the end of the fourth hour.

e. Incubate in the appropriate environmental conditions according to the media and specimen.

2. Second day (Hours 5-8)

a. Perform gram stain on any growth on plates from the spinal fluid specimens. Inoculate appropriate biochemicals for the identification of the organism. Replace plates in the appropriate incubator.

b. Screen all throat specimen plates for beta hemolytic colonies. Perform catalase tets, and perform a bacitracin susceptibility test on all catalase negative beta hemolytic colonies. If no beta hemolytic colonies are present, then report, "No beta hemolytic Streptococcus species isolated".

c. Screen all stool specimen plates for NLF's. Inoculate the NLF's to TSI's. If no NLF's are isolated, report "No enteric pathogens isolated".

3. Third day (Hours 9 & 10)

a. Record and report results of biochemicals performed on cocci from spinal fluids. Record TSI's and inoculate enteric biochemicals. Read original plates and observe for any new organisms.

b. Record and report results of bacitracin susceptibility tests performed from throat specimens.

c. Record the TSI's from the stool specimens and inoculate enteric biochemicals for each TSI.

4. Fourth day (Hours 11 & 12)

a. Record and report enteric biochemicals from the spinal fluid specimens.

b. Record and report enteric biochemicals from the stool specimens.

III. REVIEW (5 min)

A. Questions From Students

B. Review of Main Points

1. Assess level of progress for the day

2. Record all results for the specimens.

3. Clean up after the day's activities.

C. Closing Statement: As a result of this exercise you should be more aware of the type of procedures performed in an Army bacteriology laboratory. The knowledge and practical experience you have received as the result of this practical exercise will be of great assistance to you in any future assignment.

INSTRUCTIONAL HOURS

Number of instructors required - - - 8

Primary hours	72
Secondary hours	0
Total	72

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ANNEX A

Supplies and Equipment

1. Autoclave, laboratory	1 per class
2. Refrigerator	1 per class
3. Incubator, biological large (regular and CO_2)	1 per class
4. Microscope, binocular, complete	1 per student
5. Loop, inoculating, with platinum or nichrome wire	1 per student
6. Needle, inoculating, with platinum or nichrome wire	1 per student
7. Pencil, wax	1 per student
8. Gram stain reagents	7 sets per class
9. Forceps	1 per student
10. Disinfectant, germicidal and fugicidal	2 gallons per class
11. Detergent, sugical	1 gallon per class
12. Racks, test tube, metal 40-hole	1 per student
13. Racks, test tube, metal 90-hole	2 per student
14. Burner, gas, laboratory, Bunsen, or Touchmatic	1 per 2 students
15. Buckets, metal, decontamination	8 per class
16. Towels, paper	14 packages per class
17. Slides, microscopic, frosted end 25 x 75mm	1 box per student
18. Immersion oil, microscopic	1 bottle per student
19. Rack, biological staining	7 per class

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20. Tray, metal, large (non-standard)	1 per class
21. Laboratory worksheet M 08-280-433-2	1 per student
22. Swabs, sterile, in sterile plastic containers	8 per student
23. Plate canisters	2 per student
24. Mineral oil, sterile	500 ml. per class

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ANNEX B

Media and Reagents

1. Hektoen agar (HEK)	3 plates per student
2. MacConkey agar (MAC)	5 plates per student
3. Chocolate agar (Choc)	2 plates per student
4. Blood agar (SBA)	7 plates per student
5. Triple Sugar Iron agar (TSI)*	4 tubes per student
6. Indole*	1 tubes per student
7. Motility with TTC*	4 tubes per student
8. Urea, Christensen's (0.5 ml.)*	4 tubes per student
9. Lysine decarboxylase*	4 tubes per student
10. Phenylalanine*	4 tubes per student
11. ONPG (0.5 ml.)*	4 tubes per student
12. Bile Esculin Azide agar (BEA)*	1 tube per student
13. Plasma, human or rabbit, fresh or dehydrated, 0.5 ml.*	1 tube per student
14. Oxidase reagent, 1% solution	1 bottle per 4 students
15. Hydrogen peroxide, 30%	1 bottle per 4 students
16. Kovac's reagent	1 bottle per 4 students
17. 10% $FeCl_3$ solution	1 bottle per 4 students
18. Bacitracin impregnated discs	1 vial per 4 students
19. Optochin impregnated discs	1 vial per 4 students

NOTE: All plate media--15 ml. medium per plate; TSI, Phenylalanine, Motility, BEA--4 to 5 ml. per tube and slanted; all other tubed media--3 to 5 ml. per tube unless indicated.

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* 13 x 100 mm. test tubes

ANNEX C

Schedule for Preparing Organism Mixtures

Organism	Mixture Numbers
Escherichia coli	6*, 17, 18, 19, 20
Klebsiella spp.	25, 26, 27, 28
Citrobacter freundii	8*, 21, 22, 23, 24
Haemophilus spp.	5*
Salmonella spp. with H_2S	18, 26
Shigella spp.	17, 21, 25
Salmonella spp. without H_2S	22
Providencia spp.	7*, 19, 23, 27
Proteus morganii	20, 24, 28
Alpha Streptococcus spp., not group D	2*, 9, 10, 11, 12, 16
Staphylococcus aureus	4*, 12, 13
Group D gamma Streptococcus spp.	9, 13, 14, 15
Neisseria spp.	3*, 16*
Group A beta Streptococcus spp.	10, 14
Beta Streptococcus spp., not group A	11, 15

NOTE: Inoculate each organism to 50 ml. of Todd-Hewitt broth the day prior to the practical exercise.

*Inoculate to blood agar plate. These organisms will be suspended in saline as simulated spinal fluid specimens.

ANNEX D

KEY TO ORGANISM IDENTIFICATION

Key of Organism Mixtures

Mixture #	Organisms
1	No Organism
2	Alpha *Streptococcus* spp., not group D
3	*Neisseria* spp.
4	*Staphylococcus aureus*
5	*Haemophilus* spp.
6	*Escherichia coli*
7	*Providencia* spp.
8	*Citrobacter freundii*
9	Alpha *Streptococcus* spp., not group D/Group D gamma *Streptococcus* spp.
10	Alpha *Streptococcus* spp., not group D/Group A beta *Streptococcus* spp.
11	Alpha *Streptococcus* spp., not group D/Beta *Streptococcus* spp., not group A
12	Alpha *Streptococcus* spp., not group D/*Staphylococcus aureus*
13	Group D gamma *Streptococcus* spp./*Staphylococcus aureus*
14	Group D gamma *Streptococcus* spp./Group A beta *Streptococcus* spp.
15	Group D gamma *Streptococcus* spp./Beta *Streptococcus* spp., not group A
16	Alpha *Streptococcus* spp., not group D/*Neisseria* spp.
17	*Escherichia coli*/*Shigella* spp.

18 *Escherichia coli*/*Salmonella* spp. with H_2S

19 *Escherichia coli*/*Providencia* spp.

20 *Escherichia coli*/*Proteus morganii*

21 *Citrobacter freundii*/*Shigella* spp.

22 *Citrobacter freundii*/*Salmonella* spp. without H_2S

23 *Citrobacter freundii*/*Providencia* spp.

24 *Citrobacter freundii*/*Proteus morganii*

25 *Klebsiella* spp./*Shigella* spp.

26 *Klebsiella* spp./*Salmonella* spp. with H_2S

27 *Klebsiella* spp./*Providencia* spp.

28 *Klebsiella* spp./*Proteus morganii*

Swab Requirements for Each Mixture

Mixture Number	20	30	40	50	60	70	80
1	6	10	12	16	18	22	24
2	4	5	8	9	12	13	16
3	6	8	12	14	18	20	24
4	4	6	8	10	12	14	16
5	5	8	10	13	15	18	20
6	5	8	10	13	15	18	20
7	5	7	10	12	15	17	20
8	5	8	10	13	15	18	20
9	6	9	12	15	18	21	24
10	4	5	8	9	12	13	16
11	8	13	16	21	24	29	32
12	9	13	18	22	27	31	36
13	9	13	18	22	27	31	36
14	8	11	16	19	24	27	32
15	8	12	16	20	24	28	32
16	8	14	16	22	24	30	32
17	4	4	8	8	12	12	16
18	4	5	8	9	12	13	16
19	3	5	6	8	9	11	12
20	6	9	12	15	18	21	24

21	8	12	16	20	24	28	32
22	4	7	8	11	12	15	16
23	4	6	8	10	12	14	16
24	7	10	14	17	21	24	28
25	7	11	14	18	21	25	28
26	6	11	12	17	18	23	24
27	4	6	8	10	12	14	16
28	3	4	6	7	9	10	12

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Key of Specimens for Each Rack

	Spinal Fluid			Throat			Stool	
Rack #	**#1**	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**	**#8**
1	7	3	16	11	15	22	18	24
2	4	8	9	12	16	26	20	21
3	2	1	15	11	13	22	26	25
4	4	5	16	14	12	23	21	19
5	5	8	11	12	13	24	25	26
6	1	6	9	11	15	25	27	21
7	5	3	14	13	16	21	20	23
8	1	8	11	16	12	22	24	27
9	7	6	9	13	14	28	20	26
10	6	1	10	16	15	25	26	19
11	4	5	11	15	12	22	21	24
12	7	3	13	10	14	20	27	28
13	1	6	12	11	15	17	18	20
14	7	4	13	14	9	26	17	24
15	3	8	15	10	12	23	21	25
16	2	1	14	13	9	24	19	28
17	3	6	12	13	16	21	18	23
18	7	5	10	14	11	17	20	25
19	3	2	9	16	15	24	27	21
20	8	2	13	12	14	25	18	17

NOTE: For more than 20 specimens, repeat cycle numbering 21, 22, 23, etc. See page D-1 for Key for Organism Mixtures.

SSG Philip A. Salerno

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-433-1
LABORATORY SCIENCES DIVISION 018
MICROBIOLOGY BRANCH

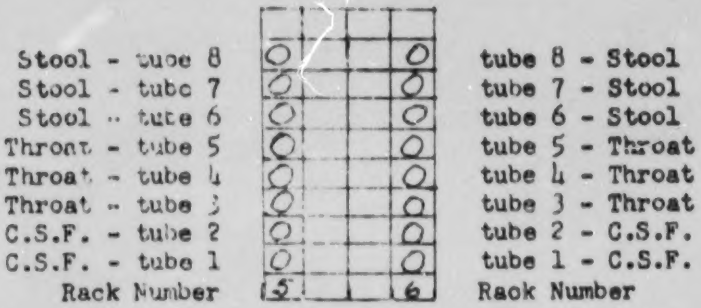
Procedure for Mixed Organism Practical Exercises

This mimeo may be used for both the Mixed Organism Practical Exercise in the fourth week of bacteriology, and the final Identification of Bacterial Exam in the fifth week. Although it is ungraded, the fourth week PE can be used as a practice for the final graded PE because both PE's are conducted in essentially the same manner. See the last page of this mimeo for details of how the final PE will be graded. Following is the objective for this practice PE:

> Given necessary materials and bacterial cultures, select proper media, correctly inoculate and streak agar plates, select proper incubation environment, correctly perform appropriate tests and stains, and correctly interpret and report results of tests and identify the bacteria to the genus and species as necessary.

FIRST DAY

1. A test tube rack of specimens will be issued to each pair of students. In that rack will be two sets of specimens of eight tubes each; one entire set (8 tubes) will be issued to each student. The tubes are arranged as shown below.


Stool - tube 8
Stool - tube 7
Stool - tube 6
Throat - tube 5
Throat - tube 4
Throat - tube 3
C.S.F. - tube 2
C.S.F. - tube 1
Rack Number
5
6
tube 8 - Stool
tube 7 - Stool
tube 6 - Stool
tube 5 - Throat
tube 4 - Throat
tube 3 - Throat
tube 2 - C.S.F.
tube 1 - C.S.F.
Rack Number

Students should use the rack number corresponding to their student number.

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*This mimeo supercedes M 08-280-433-1, 016

2. Inoculation of media

Type of specimen	Media inoculated
a. CSF ①	SBA, Choc, MAC
b. Throat	SBA ②
c. Stool	MAC, HEK

Streak each plate for isolation

①Centrifuge the two spinal fluid specimens prior to inoculation to media. Also perform a gram stain on the sediment from the centrifuged spinal fluids and turn in this report not later than the end of this day.

②Throat specimen SBA's should be stabbed in all four quadrants at least 5-10 times per plate.

3. Stack inverted MAC and HEK plates in a metal plate cylinder and place it in the incubator.

4. Place the SBA and chocolate plates in the CO_2 incubator or candle jar and incubate them.

5. Obtain two copies of PE Report Sheet (M 08-280-433-2, M 08-280-433-3) and fill in your name and rack number. One copy (M 08-280-433-3) will be returned to the instructor.

6. Clean up as directed.

SECOND DAY

1. Record the colony morphologies from the plates inoculated from spinal fluids. Perform gram stain on any growth from chocolate agar and inoculate appropriate biochemicals. (see M 08-280-432-1)

2. Record the colony morphologies from the SBA plates inoculated from throat specimens. On all beta hemolytic colonies, perform a catalase test. On the catalase negative, beta hemolytic colonies, perform a bacitracin susceptibility test. If no beta hemolytic colonies are present report, "No beta hemolytic *Streptococcus* spp. isolated".

3. Record the colony morphologies from the MAC and HEK plates inoculated from stool specimens. Inoculate all potential pathogens to TSI's. If no potential pathogens are present report, "No enteric pathogens isolated".

4. Clean up as directed. Save all plates and tube tests.

THIRD DAY

1. Record any new organism growth from the plates inoculated from the spinal fluids.

2. Continue biochemical testing and reporting of results. This is the LAST day to inoculate a test that must be incubated overnight. (see M 08-280-432-1)

3. Save all plates and tube tests. Interpret and record results of tests at the appropriate times, but since no test takes longer than 24 hours, it is permissible to read and record results of tube tests, then leave them in the test tube rack and re-incubate them with new tests. Do not attempt to read the results of the old tests after 48 hours since the prolonged incubation will cause false results. Agar plates should be refrigerated rather than incubated.

4. Clean up as directed.

FOURTH DAY

1. Report all results. This is the LAST day.

2. Turn in your report sheet for grading when finished.

3. Clean up as directed.

GRADING PROCEDURE

The final PE (Identification of Bacteria Exam) is a crucial objective exam for which the following is the objective:

Given simulated clinical specimens, correctly identify and report 90 percent of the bacteria given by properly utilizing specified identification charts.

The identification and report as well as the method of identification must be correct for 90 percent of the bacteria given. The method of identification and the reported names of the bacteria must agree with the answer key and the standards stated on the performance evaluation checklist. Briefly, the criteria stated on the checklist are as follows:

1. Reported name must agree with the answer key.

2. Name is indicated on the flow chart by the test reactions obtained.

3. Selection of tests are based on results of previous test results.

4. Tests are performed chronologically as indicated on the flow chart.

5. If a problem occurs, realize that a problem exists; then, logically re-perform the tests necessary for identification.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-434
LABORATORY SCIENCE DIVISION 038
MICROBIOLOGY BRANCH

Clinical Specimens (2 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Procedures (Basic)

PLACE: Classroom

REFERENCES:

GR 09-280-465-1
Bailey, W. R., and Scott, E. G. Diagnostic Microbiology. 4th ed. St. Louis: C. V. Mosby Company, 1974, Chapters 6, 8, 10, 11, and 13.
Lennette, E. H. and others (ed). Manual of Clinical Microbiology. 2d ed. Washington: American Society for Microbiology, 1974, Chap 6.

STUDY ASSIGNMENT: Review GR 09-280-465-1

STUDENT UNIFORM AND EQUIPMENT: Not Applicable

TOOLS, EQUIPMENT, AND MATERIALS: GR 09-280-465-1 one each per student.

PERSONNEL: One primary instructor (MOS 92B3H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, eraser, slide projector, screen; GR 09-280-465-1; LP 08-280-436-1 thru 23.

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

FIRST HOUR

I. INTRODUCTION (5 min)

A. Opening Statement: Proper initial processing of a clinical specimen is probably the single most important duty in the bacteriology laboratory. Improper technique in collecting or handling of specimens can result in failure to isolate and identify a bacterial disease-causing agent. The laboratory specialist must be able to handle each type of specimen correctly and advise others on proper methods of collection and transport.

This lesson plan supersedes LP 08-280-436, 025.

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B. Objectives

1. Given a bacteriology specimen, select the proper method of collection, processing, and media of choice for initial isolation.

2. Given a bacteriology specimen, select bacteria that may be pathogenic in that specimen.

C. Class Procedure and Lesson Tie-in

1. Demonstration of proper method for obtaining blood specimens for culture by instructor.

2. The various bacterial species studied will be isolated from simulated clinical specimens in this course.

II. EXPLANATION (70 minutes)

A. Function and Responsibilities of Personnel

1. Physicians, nurses, and ward personnel

a. Physician requests procedure

b. Nurses and ward personnel collect and deliver specimens most of the time

c. Specimens must be delivered immediately

(1) Avoids loss and/or overgrowth of organisms

(2) Use transport media, if required

d. Special precautions are taken for anaerobic bacteria

2. Laboratory personnel

a. Advise on collection and transport techniques

b. Collect certain specimens (e.g. blood cultures)

c. Process specimen as requested

d. Note any deficiencies in collection and/or transport

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  - e. Log in specimen
  - f. Route and process according to SOP
  - g. Report results in timely manner

B. Proper completion of request form

  1. Complete patient identification
  2. Clear designation of specimen source
  3. Procedures desired
  4. Antibiotic therapy of patient (if any)
  5. Time and date collected
  6. Place of origin (e.g. ward, clinic, dispensary, etc.)
  7. Physician's signature
  8. Attach to or label specimen

C. Shipping and Receiving Bacterial Specimens

  1. Routine packing
    - a. Screwcapped tubes and bottles
      - (1) Lips intact
      - (2) Cap liners intact
      - (3) Caps secured with tape
      - (4) Sealed with paraffin
    - b. Absorbent cotton soaked with disinfectant
    - c. Double mailing container
    - d. Request form with necessary information
    - e. Outer mailing label

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2. Special-handling packing
    a. Highly infectious specimens
    b. Heat-sealed ampule
    c. Formalin - approximately 4 percent
3. Specific
    a. Follow SOP
    b. Organisms for identification
        (1) Viable, pure culture on agar slant
        (2) Information included
            (a) Growth characteristi[illegible]
            (b) Colony morphology
            (c) Results of tests performed
4. Receiving
    a. Specimen broken
        (1) Autoclave
        (2) Request second specimen
    b. Specimen intact - process as requested

NOTE: Have students follow lecture with GR 09-280-465-1.

D. Urine
1. Diseases
    a. Urethritis (anterior tract)
    b. Cy[illegible]itis (bladder)
    c. Nephritis (kidney)

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2. Indigenous flora - none, except normal genital flora

3. Predominant pathogens

   a. Enterics

   b. *Pseudomonas* species

   c. *Staphylococcus aureus*

   d. *Candida* species

4. Collection

   a. Routine technique - midstream, clean-catch method

      (1) External urethral orifice cleansed

      (2) Use sterile capped container

      (3) Urine collected aseptically after urethra flushed by first portion of urine

   b. Catheterization method - by physician, nurse or ward personnel

      (1) Catheter tube inserted into bladder through external orifice

      (2) Urine collected in sterile container through tube

   c. Bladder tap - by physician (suprapubic aspirate)

      (1) Needle punctures directly into bladder

      (2) Urine aspirated into syringe

5. Transport to the laboratory

   a. Immediately (process within 2 hours)

   b. Refrigerate within 10 minutes for up to 24 hours if unable to process immediately

6. Laboratory processing

   a. Media

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- (1) Sheep blood agar
- (2) MacConkey agar

b. Inoculation

- (1) Calibrated loop method
  - (a) Choice method
  - (b) Deliver 0.01 or 0.001 ml to plate
  - (c) Streak 2 or 3 directions for confluent growth
  - (d) Provides ease of performance and accuracy
  - (e) Number of colonies multiplied by the dilution factor equals number of colonies per ml of urine
- (2) Tube dilution method
  - (a) Dilute urine in sterile water (use pipette)
  - (b) Deliver 0.1 ml of 1:100 dilution and/or 1:1000 dilution to plate
  - (c) Streak 2 or 3 directions for confluent growth
- (3) Poured plate method
- (4) Uri-cult

SECOND HOUR

E. Blood

1. Diseases
   - a. Bacteremia - the transient invasion of the bloodstream by bacteria
   - b. Septicemia - the presence in the bloodstream of actively multiplying bacteria and their toxins which usually cause chills, fever, prostration, etc.
2. Indigenous flora - none

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3. Predominant pathogens
    - a. *Escherichia coli*
    - b. *Staphylococcus aureus*
    - c. Streptococci
    - d. *Psuedomonas* species
    - e. *Bacteroides* and other anaerobes
    - f. *Haemophilus* species
    - g. *Neisseria* species

4. Collection
    - a. Collect at peak of chills or fever
    - b. Draw multiple specimens
    - c. Aspirate by venipuncture (usually 5 to 10 ml)
    - d. Use strict aseptic technique
    - e. Materials
        - (1) Blood culture bottles - blood culture broth (commercially prepared, evacuated, with CO_2 atmosphere)
            - (a) Anticoagulant (small amount) SPS
            - (b) Inhibitor of antibiotics
        - (2) Disinfectant/cleansing solutions
        - (3) Double-needle tubing
        - (4) Needle and syringe
    - f. Technique
        - (1) Label 2 bottles (aerobic and anaerobic)

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(2) Select venipuncture site

(3) Cleanse venipuncture site and bottle stoppers

(a) Surgical soap

(b) Tincture of iodine - 1 minute

(c) 70 percent alcohol

(d) Tincture of iodine

(e) 70 percent alcohol

(f) Cover with sterile gauze - do not palpate and contaminate after this cleansing

(4) Perform venipuncture

(5) Withdraw 5 to 20 ml of blood with collecting unit

NOTE: A final broth concentration of 1:10 to 1:20 is desired.

5. Laboratory processing

a. Aerate one bottle

b. Incubation

(1) 35 C

(2) Routine - up to 14 days

(3) Special request - at least 21 days (e.g. Brucella)

c. Daily macroscopic observation

(1) Turbidity

(2) Gas bubbles

d. Subculture and Gram stain

(1) 24 hours

(2) 48 hours or 72 hours

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        (3) 14 days

        (4) Anytime bottle appears positive

    e. Identify and report all isolates

F. Sputum

1. Diseases
    - a. Viral - discussed in virology
    - b. Fungal - discussed in mycology
    - c. Bacterial
        - (1) Pneumonia
        - (2) Tuberculosis

2. Indigenous flora - none usually (beware of contaminants picked up during passage of sputum through the URT)

3. Predominant pathogens
    - a. Staphylococcus aureus
    - b. Haemophilus influenzae
    - c. Various enterics
    - d. Pseudomonas aeruginosa
    - e. Streptococcus pneumoniae
    - f. Mycobacterium tuberculosis
    - g. Streptococcus pyogenes
    - h. Various anaerobes

4. Collection
    - a. Sterile, wide-mouthed, capped container
    - b. Collect as soon as patient awakes in morning
    - c. More than one specimen for TB

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5. Laboratory processing

   a. Must be received without undue delay (1 to 2 hours)

   b. Refrigerate 1 to 3 hours if delay unavoidable

   c. Macroscopic examination (color, texture)

   d. Microscopic examination - Gram stain

   e. Media

      (1) Sheep blood agar

      (2) MacConkey agar

   f. Inoculation

      (1) Swab

      (2) Loop

      (3) Streak for isolation

   g. Incubation - routine

   h. Identify and report potential pathogens

G. Wounds and Exudates (Eye, Ear, Lesion, etc.)

1. Diseases

   a. Conjunctivitis

   b. Otitis externa

   c. Wounds

   d. Abscesses

   e. Burns

2. Indigenous flora - none, except contaminating skin flora

3. Predominant pathogens

   a. Staphylococcus aureus

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b. *Streptococcus pyogenes*

c. *Streptococcus pneumoniae*

d. *Pseudomonas aeruginosa*

e. *Haemophilus aegyptius*

f. *Haemophilus influenzae*

g. Anaerobic bacteria

h. Enterics

4. Collection

a. Sterile swab with transport media

b. Anaerobic collection devices

(1) Closed syringe

(2) Gassed-out tubes

5. Laboratory processing

a. Gram stain

b. Anaerobes - STAT

c. Aerobes - refrigerate if delay is unavoidabel (3 to 5 hours)

d. Media

(1) Sheep blood agar

(2) MacConkey agar

(3) Chocolate agar (usually unnecessary on wounds and abscesses)

(4) Thioglycollate broth

(5) Anaerobic or other special media as required by SOP

e. Incubation

(1) 35 C

(2) 24 to 72 hours; observe every 24 hours

(3) Increased CO_2 for SBA and chocolate

(4) Anaerobe setup for anaerobes

f. Identify and report all isolates

H. Questions from Students

III. SUMMARY

A. Review of Main Points

1. Function and responsibilities of personnel

2. Request form completion

3. Shipping and receiving bacterial specimens

4. Urine

5. Blood

6. Sputum

7. Wounds and exudates

B. Closing Statement: Collecting and processing bacteriological specimens are two of the most vital steps taken in attempting to recover pathogenic organisms from clinical specimens. Your ability to do these procedures properly enables the physician to give better care for the patient and contributes to the efficiency of your laboratory.

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ANNEX A
INSTRUCTIONAL AIDS

LP 08-280-434
038

Magnetic Placards

MP 08-280-436-1	CLINICAL SPECIMENS
MP 08-280-436-2	FUNCTION & RESPONSIBILITIES OF PERSONNEL
MP 08-280-436 3	PHYSICIANS, NURSES, & WARD PERSONNEL
MP 08-280-436-4	LABORATORY PERSONNEL
MP 08-280-436-5	SHIPPING & RECEIVING SPECIMENS
MP 08-280-436-6	GENERAL
MP 08-280-436-7	ROUTINE PACKING
MP 08-280-436-8	SPECIAL-HANDLING PACKING
MP 08-280-436-9	SPECIFIC
MP 08-280-436-10	RECEIVING
MP 08-280-436-11	COLLECTION & PROCESSING OF SPECIMENS
MP 08-280-436-12	EXUDATE
MP 08-280-436-13	DESCRIPTION
MP 08-280-436-14	COLLECTION
MP 08-280-436-15	PROCESSING
MP 08-280-436-16	SPUTUM
MP 08-280-436-17	BODY FLUIDS
MP 08-280-436-18	URINE
MP 08-280-436-19	CLEAN CATCH
MP 08-280-436-20	CATHETERIZATION
MP 08-280-436-21	BLADDER TAP
MP 08-280-436-22	CYSTOSCOPIC
MP 08-280-436-23	BLOOD

NOTE: These placards are used at the main headings.

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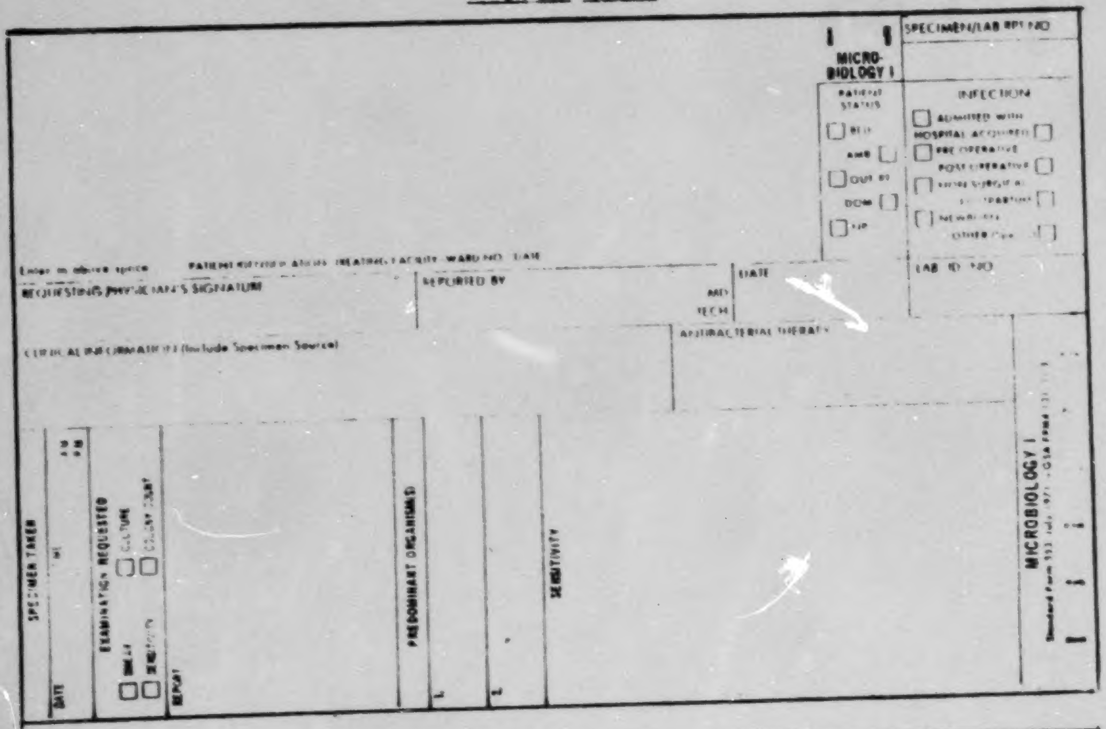
SFC Lyle J. Sorensen 1161

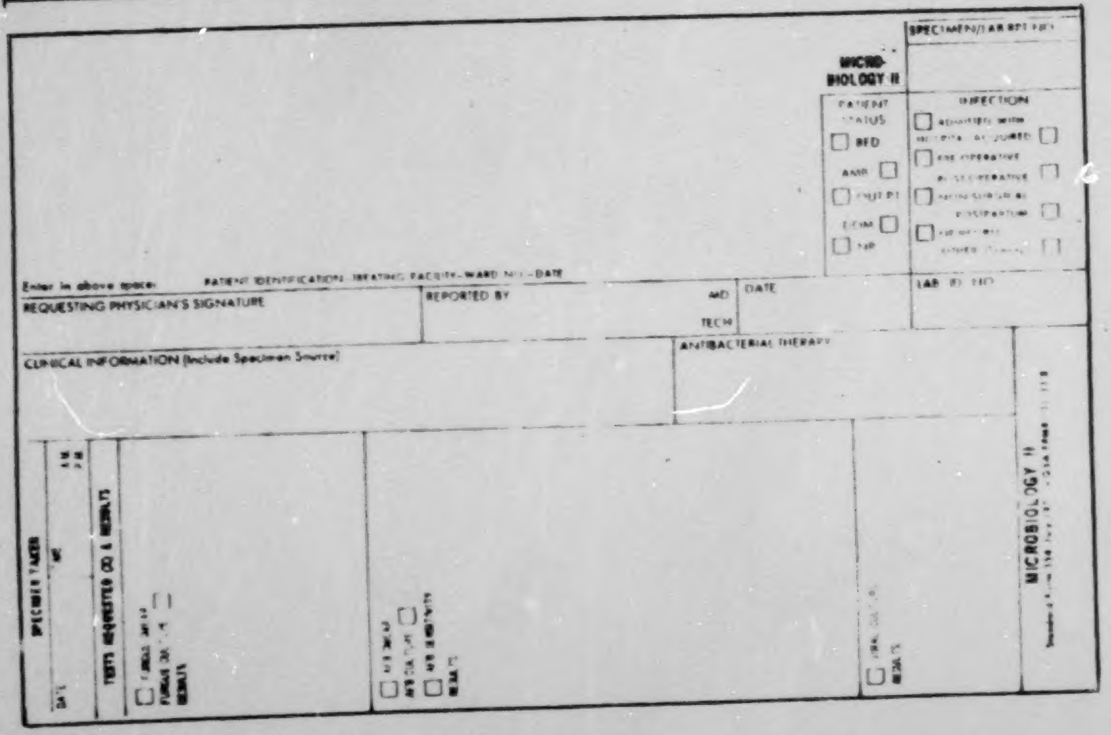
ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
MICROBIOLOGY BRANCH

*M 08-280-434-1
046

Request Forms



SPECIMEN/LAB RPT NO
MICROBIOLOGY I
PATIENT STATUS
☐ BED
AMB ☐
☐ OUT PT
DOM ☐
☐ NP
INFECTION
☐ ADMITTED WITH
HOSPITAL ACQUIRED ☐
☐ PRE OPERATIVE
POST OPERATIVE ☐
☐ [illegible]
[illegible] ☐
☐ NEWBORN
OTHER ☐
Enter in above space: PATIENT IDENTIFICATION (TREATING FACILITY-WARD NO.-DATE)
REQUESTING PHYSICIAN'S SIGNATURE
REPORTED BY
MD
TECH
DATE
LAB ID NO
CLINICAL INFORMATION (Include Specimen Source)
ANTIBACTERIAL THERAPY
SPECIMEN TAKEN
DATE
TIME
AM
PM
EXAMINATION REQUESTED
☐ SMEAR
☐ CULTURE
☐ SENSITIVITY
☐ COLONY COUNT
REPORT
PREDOMINANT ORGANISM(S)
1.
2.
SENSITIVITY
MICROBIOLOGY I
Standard Form 553 July 1971 GSA FPMR [illegible]



SPECIMEN/LAB RPT NO
MICROBIOLOGY II
PATIENT STATUS
☐ BED
AMB ☐
☐ OUT PT
DOM ☐
☐ NP
INFECTION
☐ ADMITTED WITH
HOSPITAL ACQUIRED ☐
☐ PRE OPERATIVE
POST OPERATIVE ☐
☐ [illegible]
POSTPARTUM ☐
☐ NEWBORN
OTHER ☐
Enter in above space: PATIENT IDENTIFICATION (TREATING FACILITY-WARD NO.-DATE)
REQUESTING PHYSICIAN'S SIGNATURE
REPORTED BY
MD
TECH
DATE
LAB ID NO
CLINICAL INFORMATION (Include Specimen Source)
ANTIBACTERIAL THERAPY
SPECIMEN TAKEN
DATE
TIME
AM
PM
TESTS REQUESTED (X) & RESULTS
☐ FUNGUS SMEAR
FUNGUS CULTURE ☐
RESULTS
☐ AFB SMEAR
AFB CULTURE ☐
☐ AFB SENSITIVITY
RESULTS
☐ VIRAL CULTURE
RESULTS
MICROBIOLOGY II
Standard Form 554 July 1971 GSA FPMR [illegible]

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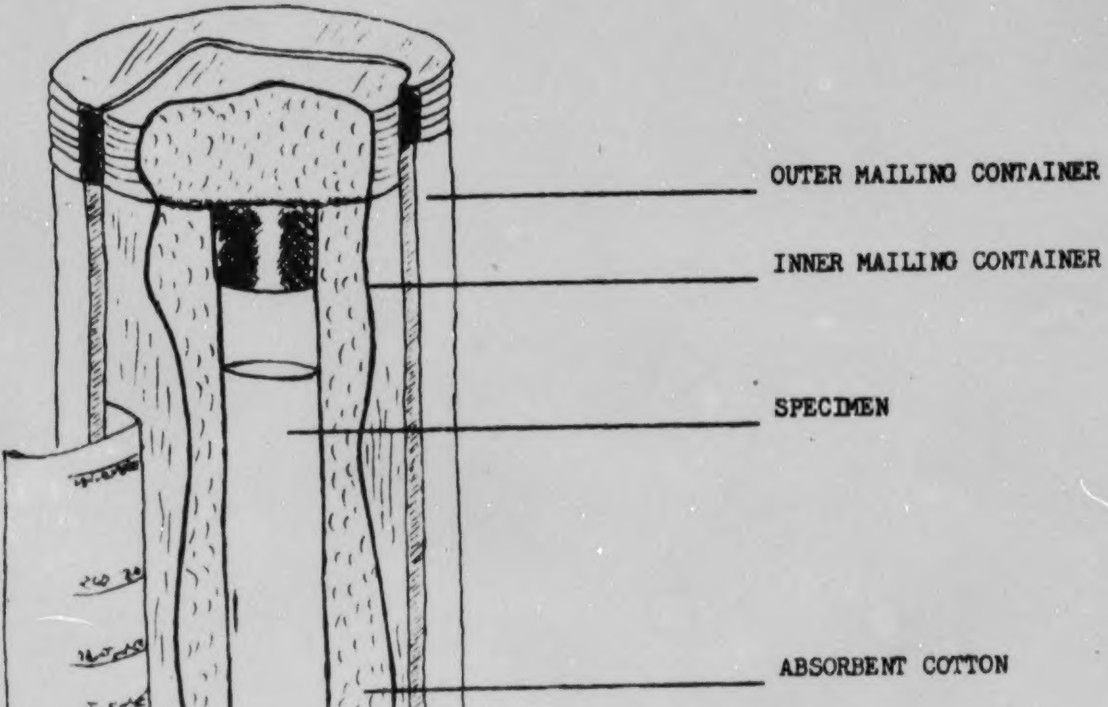
SFC Lyle J. Sorensen

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
MICROBIOLOGY BRANCH

*M 08-280-434-2
046

SPECIMEN CONTAINERS

Routine Packing


OUTER MAILING CONTAINER
INNER MAILING CONTAINER
SPECIMEN
ABSORBENT COTTON
REQUEST FORM

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*This mimeo supersedes M 08-280-434-2, 025.

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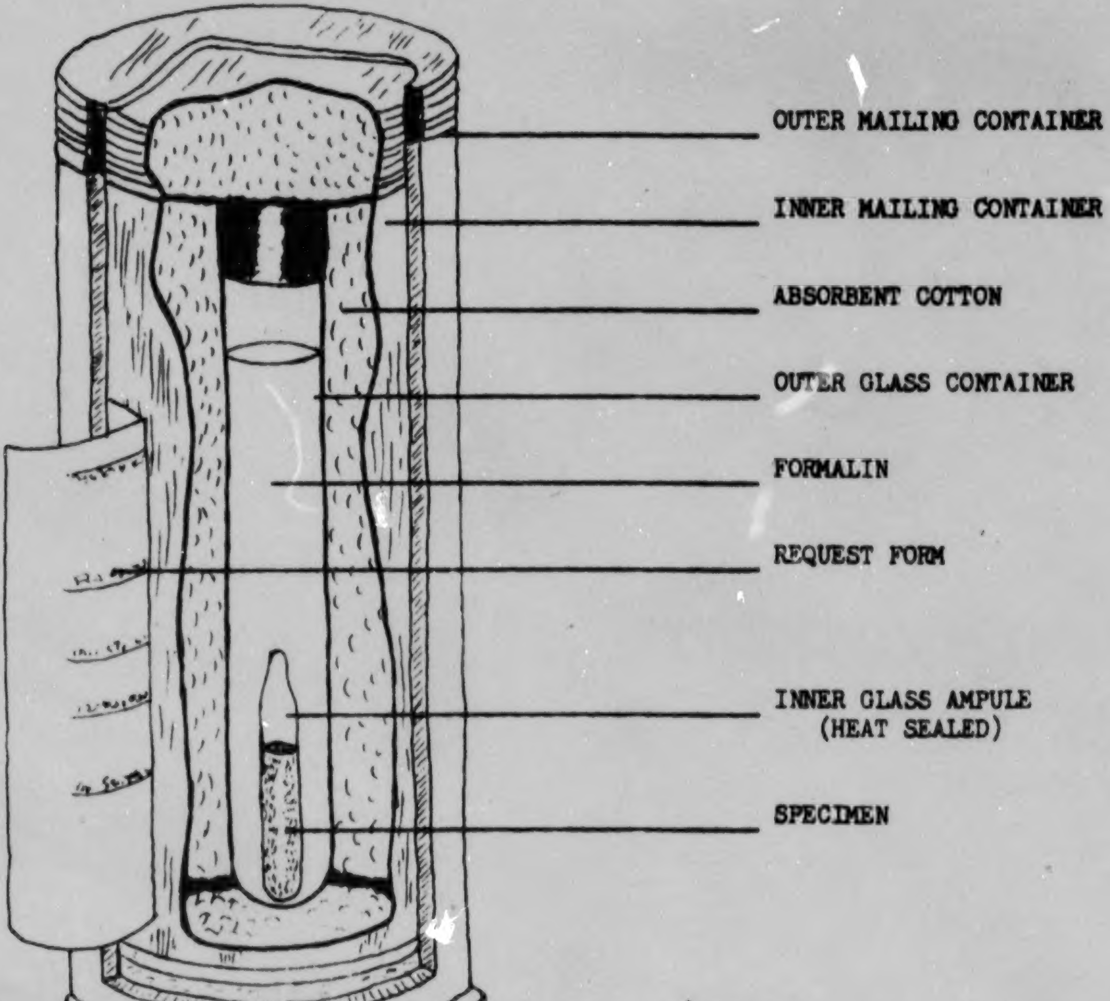
SPECIMEN CONTAINERS

Special-handling Packing: Highly Infectious Specimens


OUTER MAILING CONTAINER
INNER MAILING CONTAINER
ABSORBENT COTTON
OUTER GLASS CONTAINER
FORMALIN
REQUEST FORM
INNER GLASS AMPULE (HEAT SEALED)
SPECIMEN

1198

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-435
LABORATORY SCIENCES DIVISION 068
MICROBIOLOGY BRANCH

The Cerebrospinal Fluid (CSF) - 1 hour

COURSE PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic) course

PLACE: Classroom

REFERENCES:

Bailey, W. R., and Scott, E. G. Diagnostic Microbiology. 4th ed. St. Louis: C. V. Mosby Company, 1974.
Lennette, E. H. and others (ed). Manuel of Clinical Microbiology. 2nd ed. Washington: American Society for Microbiology, 1974.

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: N/A

TOOLS, EQUIPMENT AND MATERIALS: N/A

PERSONNEL: One primary instructor (MOS 92B30)

INSTRUCTIONAL AIDS: Magnetic placards, chalk, chalkboard and eraser

TROOP REQUIREMENTS: N/A

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Conference

I. INTRODUCTION (5 min)

A. Opening Statement: As laboratory technicians, you will be required to expedite some laboratory samples. One of these samples will be the cerebrospinal fluid (CSF). The CSF sample is one in which immediate handling and processing is very important to the well-being of the patient. Bacteriological examination of the CSF is an essential step in diagnosing cases of possible meningitis.

B. Objectives:

1. Given a cerebrospinal fluid specimen, select the correct procedure for processing that specimen.

2. From a list of organisms, select those that can be pathogenic

---

*This lesson plan supersedes LP 08-280-435, 046.

in spinal fluid.

3. Given identifying characteristics, select the characteristics that are used in the identification of Haemophilus influenzae to include the use of chocolate agar.

C. Class Procedure and Lesson Tie-in:

A practical exercise using CSF specimens will follow this lecture.

II. EXPLANATION (30 min)

A. Description of Cerebrospinal Fluid

1. Protective body fluid

a. Surrounds the meninges

b. Normally free of micoorganisms

2. Normal cerebrospinal fluid

a. Macroscopic appearance - clear

b. Has no segmented neutrophils

c. Has approximately 2/3 of glucose of blood

3. Bacterial infected spinal fluid

a. Macroscopic appearance - cloudy, white

(1) Usually marked increase in WBC's

(2) Bacteria[illegible]owth

(3) May contain RBC's

b. Usually decrease in glucose level

c. Disease called bacterial meningitis

B. Collection of Cerebrospinal Fluid

1. Specimen collected by physician

a. Collection by lumbar puncture

  - b. Strict aseptic technic must be maintained
  - c. Three portions of CSF normally collected (Tube #1 to Bacti)
  - d. Specimens should be submitted to sterile screw cap test tubes

NOTE: If only one specimen is submitted to laboratory, it should go to bacteriology first so that a portion may be removed aseptically.

2. Specimen should be transported to laboratory as soon as possible

C. Processing of Cerebrospinal Fluid

1. CSF specimens must be processed as soon as possible after being received in the laboratory

NOTE: Because of the possible clinical importance of microorganisms in the CSF, the specimen should be handled as a 'STAT' (IMMEDIATELY)

2. Concentrate CSF by centrifugation at 2500 RPM for 15 minutes
3. Aseptically, transfer the supernatant into another sterile test tube
4. Aseptically, inoculate sediment to media
    - a. Sheep blood agar
    - b. Chocolate agar
    - c. MacConkey's agar
5. Perform gram stain on remaining sediment
6. Incubate media and supernatant appropriately
7. India ink preparations may be performed to demonstrate fungal infections
8. Hold all cultures for 72 hours (5 - 7 days when antibiotics had been given before specimen was obtained) before discarding and reporting as no growth

D. Microbial agents Commonly Involved in CSF Meningitis

1. Bacterial

    a. *Haemophilus influenzae*
    b. *Neisseria meningitidis*
    c. *Streptococcus* (various species)
    d. Family Enterobacteriaciae
    e. *Listeria monocytogenes*
  2. Fungal infections (Primarily *Cryptococcus neoformans*)
  3. Various viral agents
  4. Protozoan parasites

E. Identification Characteristics of *Haemophilus influenzae*

  1. Very fastidious microorganism
  2. Require X and V factors for growth
    a. Whole blood contains X factor which is heat stable (Explain)
    b. Whole blood contains V factor which is heat labile (Explain)
  3. Cultural characteristics
    a. Requires enriched medium
    b. Requires increased CO_2
    c. Optimum temperature 35 - 37 degrees C
    d. Grows poorly on sheep blood agar
      (1) Antibodies present in blood
      (2) Satellite phenomenon
        (a) *Staphylococcus* species
        (b) *Neisseria* species
    e. Medium of choice chocolate agar
      (1) Highly enriched media

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(2) Contains basal medium and heated blood

(a) Blood provides X factor

(b) V factor replaced by addition of yeast extract or commercial Iso - Vitalex enrichment

(c) Heated blood is brown, giving the medium chocolate color

4. Colony morphology on chocolate agar (after 18 - 24 hours, 37°C)

   a. Small

   b. Colorless

   c. Moist colonies with distinct odor

5. Catalase positive

6. Main identification tests are X and V strips

7. Gram morphology

   a. Gram negative

   b. Bacillus (coccobacilli)

   c. No predominent arrangement

8. Pathogenicity

   a. Bacterial meningitis in children under 3 years old

   b. Rarely found in adults

   c. Serological type b most common

F. Question from Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Description of cerebrospinal fluid

2. Collection of cerebrospinal fluid

3. Processing of cerebrospinal fluid

4. Microbial agents commonly involved in CSF meningitis

5. Identification characteristics of Haemophilus influenzae

B. Closing Statement: The rapid determination of the presence or absence of bacteria in a CSF specimen is of utmost importance to the physician. Rapid identification of bacteria in a CSF specimen will allow the physician to treat the patient with the most effective antibiotics, and therefore improve the probability of the patient's recovery.

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ANNEX A

INSTRUCTIONAL AIDS

Magnetic Placards

1. Description of Cerebrospinal Fluid
2. Collection of Cerebrospinal Fluid
3. Processing of Cerebrospinal Fluid
4. Microbial Agents of CSF Meningitis
5. Haemophilus influenzae
6. Neisseria Meningitidis
7. Streptococcus
8. Enterobacteriaceae
9. Listeria Monocytogenes
10. Characteristics of Haemophilus influenzae

SSO Philip A. Salerno

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY #M 08-280-436-1
LABORATORY SCIENCES DIVISION 048
MICROBIOLOGY BRANCH

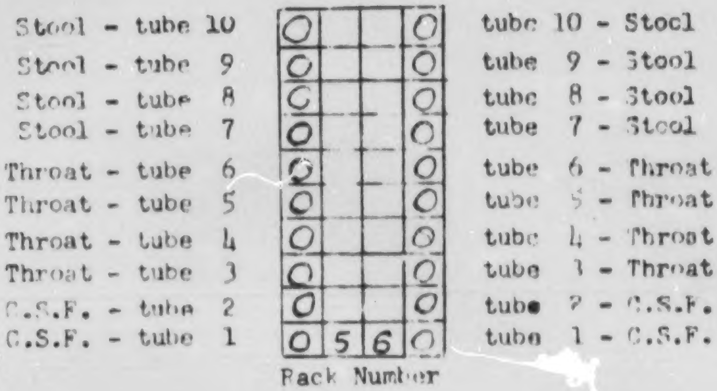
Procedure for Final Identification of Bacteria Exam

This mimeo may be used for the final Identification of Bacteria Exam in the fifth week of bacteriology. This exam is a crucial objective and on the last page of this mimeo you can find the details of how this PE will be graded. The following is the objective for this PE:

> Given simulated spinal fluid specimens, perform initial gram stains and report the presence or absence of bacteria to 100% accuracy. Given simulated throat, stool, and cerebrospinal fluid specimens, correctly identify and report pathogens present by utilizing necessary material provided and specified identification charts, to an accuracy of 80 percent.

FIRST DAY

1. A test tube rack of specimens will be issued to each pair of students. In that rack will be two sets of specimens of ten tubes each; one entire set (10 tubes) will be issued to each student. The tubes are arranged as shown.


Stool - tube 10
Stool - tube 9
Stool - tube 8
Stool - tube 7
Throat - tube 6
Throat - tube 5
Throat - tube 4
Throat - tube 3
C.S.F. - tube 2
C.S.F. - tube 1
5
6
Rack Number
tube 10 - Stool
tube 9 - Stool
tube 8 - Stool
tube 7 - Stool
tube 6 - Throat
tube 5 - Throat
tube 4 - Throat
tube 3 - Throat
tube 2 - C.S.F.
tube 1 - C.S.F.

Students should use the rack number corresponding to their student number.

2. Inoculation of media

Type of specimen	Media inoculated
a. CSF①	SBA, Choc, MAC
b. Throat	SBA②
c. Stool	MAC, HEK

Streak each plate for isolation

①Centrifuge the two spinal fluid specimens prior to inoculation to media. Also perform a gram stain on the sediment from the centrifuged spinal fluids and turn in this report not later than the end of this day.

②Throat specimen SBA's should be stabbed in all four quadrants at least 5-10 times per plate.

3. Stack inverted MAC and HEK plates in a metal plate cylinder and place it in the incubator.

4. Place the SBA and Chocolate plates in the CO_2 incubator or candle jar and incubate them.

5. Obtain two copies of PE Report Sheet (M 08-280-433-2, M 08-280-433-3) and fill in your name and rack number, One copy (M 08-280-433-3) will be returned to the instructor.

6. Clean up as directed.

SECOND DAY

1. Record the colony morphologies from the plates inoculated from spinal fluids. Perform gram stains and inoculate the appropriate biochemicals. (See M 08-280-432-1)

2. Record the colony morphologies from the SBA plates inoculated from throat specimens. On all beta hemolytic colonies, perform a catalase test. On the catalase negative, beta hemolytic colonies, perform a bacitracin susceptibility test. If no beta hemolytic colonies are present report, "No beta hemolytic Streptococcus spp. isolated".

3. Record the colony morphologies from the MAC and HEK plates inoculated from stool specimens. Inoculate all potential pathogens to TSI's. If no potential pathogens are present report, "No enteric pathogens isolated".

4. Clean up as directed. Save all plates and tube tests.

THIRD DAY

1. Record any new organism growth from the plates inoculated from the spinal fluids.

2. Continue biochemical testing and reporting of results. This is the LAST day to inoculate a test that must be incubated overnight. (See M 08-280-432-1)

3. Save all plates and tube tests. Interpret and record results of tests at the appropriate times, but since no test takes longer than 24 hours, it is permissible to read and record results of tube tests, then leave them in the test tube rack and re-incubate them with new tests. Do not attempt to read the results of the old tests after 48 hours since the prolonged incubation will cause false results. Agar plates should be refrigerated rather than incubated.

4. Clean up as directed.

FOURTH DAY

1. Report all results. This is the LAST day.

2. Turn in your report sheet for grading when finished.

3. Clean up as directed.

GRADING PROCEDURE

The identification and report as well as the method of identification must be correct for 80 percent of the bacteria given. The method of identification and the reported names of the bacteria must agree with the answer key and the standards stated on the performance evaluation checklist. Briefly, the criteria stated on the checklist are as follows:

1. Reported name must agree with the answer key.

2. Name is indicated on the flow chart by the test reactions obtained.

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3. Selection of tests are based on results of previous test results.

4. Tests are performed chronologically as indicated on the flow chart.

5. If a problem occurs, realize that a problem exists; then, logically re-perform the tests necessary for identification.

1209

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY
LABORATORY SCIENCES DIVISION
MICROBIOLOGY BRANCH

*LP 08-280-437
048

Kirby-Bauer Practical Exercise - 2 hours

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic)

PLACE: Classroom

REFERENCES: All previous references

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: See Annex A thru E

PERSONNEL: Eight instructors (MOS 92B3H)

INSTRUCTIONAL AIDS: See Annex A thru E

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Practical exercise

I. INTRODUCTION (5 min)

A. Opening Statement: This exercise is designed to acquaint you with one of the most common procedures performed in a clinical bacteriology laboratory, the Kirby-Bauer single disk method of antibiotic susceptibility testing. Selection of colonies for testing, preparation of the inoculum and plates, and measuring and interpreting zone sizes are standardized throughout the United States and must be performed properly for the test to be valid.

B. Objective: Given bacterial cultures and necessary materials, perform, interpret and report results of the Kirby-Bauer susceptibility test, as demonstrated in class.

C. Class Procedure and Lesson Tie-in: The information presented in the Kirby-Bauer lecture and information from GR 09-280-465-1 will be used to perform the tests in this PE.

II. APPLICATION: (90 min)

A. Instructors' Activities

1. Divide the class into two student of teams

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*This lesson plan supersedes LP 08-280-437, 046

2. Equipment and materials issued (see Annexes A and B)
3. Inoculum prepared and issued (see Annexes D, E, and the Protocol)
4. Daily instruction given in a step-by-step fashion
5. Previous day's activities reviewed
6. Questions answered
7. Corrections made
8. Maintain laboratory safety
9. Exercise classroom control
10. Critique the practical exercise (after completion)

B. Students' Activities

1. Aseptic technique maintained
2. Prepare inoculum
3. Prepare plates
4. Read and record results
5. Culture materials discarded
6. Clean-up as directed
7. Equipment stored properly

C. Organization of Instructional Hours

1. First day (45 min) See Annexes A, B, C, & E
    - a. Compare inoculum to barium chloride standards
    - b. Dilute inoculum using sterile saline turbidity to desired
    - c. Inoculate plate and streak for confluent growth
    - d. Dispense appropriate antibiotic disks to plate
    - e. Insure that each disk has good contact with the agar
    - f. Incubate cultures

        g. Clean-up as directed

    2. Second day (45 min)

        a. Observe plates for zones of inhibition.

        b. Measure zone sizes

        c. Compare measurements to chart in GR 09-280-465-1

        d. Report results

        e. Discard cultures

        f. Clean-up as directed

D. Questions From Students

III. SUMMARY (5 min)

A. Review of Main Points

    1. Preparation of inoculum

    2. Inoculation of plates

    3. Application of disks

    4. Interpretation of results

B. Closing Statement: The performance of the Kirby-Bauer test must remain an exact and controlled procedure. If the test is not performed with adherence to strict controls the results may be incorrect. Proper performance and interpretation of the Kirby-Bauer test will lead to better selection of antimicrobial agents for the treatment of infections.

ANNEX A

INSTRUCTIONAL AIDS

Tools Equipment and Materials

1. Autoclave, laboratory	1 per class
2. Incubator	1 per class
3. Swabs, sterile	1 per student
4. Rack, wire 90-hole	1 per 2 students
5. Sterile Dispopet	1 per student
6. Bulb, rubber	1 per student
7. Tape, masking	1 roll per class
8. Towels, paper	7 pg per class
9. Disinfectant, germicidal	1 gal per class
10. Detergent, surgical	7 bottles per class
11. Marker, wax	1 per student
12. Forceps, flat tips	1 per student
13. Ruler, plastic, metric	1 per student
14. Bucket, metal, decontamination	4 per class
15. Dispensers, antibiotic disk, multiple	8 per class
16. Pad, inked	1 per class
17. Stamp, rubber	2 per class
18. Test tubes, glass 13 X 100 mm	2 per student
19. Enclosures, test tube	1 per class
20. Parafilm	1 box per class
21. Bottle, screw cap, prescription	1 box per class
22. Burner, gas, touchmatic with tubing	1 per student
23. Loop inoculating	1 per class

ANNEX B

Media and Reagents

1. Mueller Hinton agar, 150 mm	1 plate per student
2. Mueller Hinton broth, 2 ml*	1 tube per student
3. Sterile normal saline, 5 ml*	1 tube per student
4. Barium chloride standard, 2 ml*	8 tubes per class
5. Todd-Hewitt broth, 50 ml	2 bottles per class
6. Antibiotic impregnated disks (see Annex E)	12 per student
7. Alcohol, 50 ml	8 bottles per class

* 13 X 100 mm test tubes

ANNEX C

Antibiotic Impregnated Disks

Gram positive battery:

1. Amikacin
2. Cephalothin
3. Choloramphenicol
4. Erythromycin
5. Clindamycin
6. Gentamicin
7. Methicillin
8. Penicillin G
9. Tetracycline
10. Vancomycin
11. Kanamycin or Ampicillin
12. Neomycin or Bacitracin

Gram negative battery:

1. Amikacin
2. Ampicillin
3. Carbenicillin
4. Cephalothin
5. Chloramphenicol
6. Colistin
7. Gentamicin
8. Tetracycline
9. Tobramycin
10. Trimethoprim - sulfamethoxazole
11. Nitrofurantoin or Nalidixic acid
12. Kanamycin

ANNEX D

Organisms to be inoculated

1. *Staphylococcus aureus*
2. *Providencia* species

ANNEX E

Procedure for Preparation of Practical Exercise

A. Materials

1. Two bottles of Todd-Hewitt broth (50 ml per bottle)
2. Burner with tubing
3. Inoculating loop
4. Sheep blood agar cultures
    a. *Staphylococcus aureus*
    b. *Providencia* species
5. 90-hole test tube rack (one per group of two students)
6. Sterile Dispopet
7. Rubber bulb
8. Masking tape
9. Felt tip marker
10. Sterile glass tubes with closures
11. Disinfectant

B. Preparation procedure

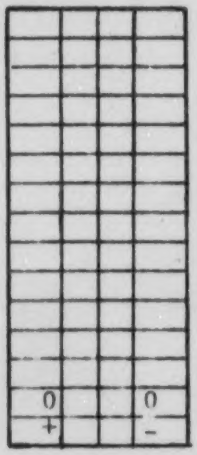
1. Day prior to PE
    a. Inoculate Todd-Hewitt bottle with organisms from appropriate culture plates. Insure that the bottles are correctly labeled.
    b. Incubate the inoculated bottles
    c. Prepare barium chloride standards
    d. Check the antibiotic disk dispensers to insure that enough disks are available for the exercise
2. Day of PE
    a. Inoculate Mueller Hinton broth tubes two hours prior to class, using sterile Dispopet and rubber bulb to add Todd-Hewitt broth with organisms to each tube

b. Add five drops of Todd-Hewitt broth inoculated with Staphylococcus aureus to tubes of Mueller Hinton broth (one tube per two students)

c. Add ten drops of Todd-Hewitt broth inoculated with Providencia species to tubes of Mueller Hinton broth (one tube per two students)

d. Incubate the tubes for two hours. Insure that growth in the Mueller Hinton broth is heavy enough so that turbidity is greater than that of the barium chloride standards

e. Place the tubes into an appropriately labeled 90-hole rack (see Protocol)



Gram positive tube
Tape label

Gram negative tube

90-hole rack

NOTE: One rack arranged in this manner is needed for every two students. Care should be taken to arrange the organisms exactly as indicated.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY M 08-280-438
LABORATORY SCIENCE DIVISION 028
MICROBIOLOGY BRANCH

Procedure for Cerebrospinal Fluid- Practical Exercise

First Day

1. The class will be divided into groups of two students each. Each group will be issued six specimens each in a 90 hole rack as indicated in figure one (1) below. Each student in the group will choose either A or B.

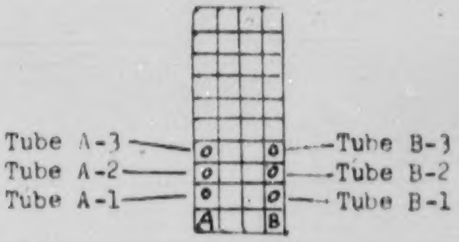


Figure 1

2. Each student will mark his specimens with the proper specimen number and his student number.

3. The student will then centrifuge each CSF specimen for 15 minutes at 2,500 RPM. The supernatant will be poured into a sterile tube and incubated. Aseptically remove some of the sediment and make two gram stain slides. Stain one of the smears and save the other slide for future reference. Record your results on the last page of this mimeo.

4. Each student will obtain three sheep blood agar (SBA), three Chocolate agar (Choc), and three MacConkey (MAC) plates, and label one of each plate for each specimen. The label should include students name, date, and culture number.

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5. Using aseptic technique, inoculate each of the sediments to a SBA, Choc, and MAC plate. Use an inoculating loop or sterile pipet to transfer a portion of the sediment to the media, then streak the media for isolation.
6. Incubate the Choc and SBA plates by placing them upside down in a metal canister and place them in the CO_2 incubator. Place the MAC plates upside down in a metal canister and incubate at 37°C, regular oxygen tension. Place the supernatants in a 90 hole rack and incubate at 37°C.
7. Clean up as directed.

Second Day

1. Remove cultures from both incubators and observe the colony morphology. Record the colony morphology on the last page of this mimeo. Also record the results that your partner gets for his specimens in this practical exercise.
2. Gram stain any growth that is on the Chocolate agar plates. If the original gram stain was negative and there is growth on the plates, gram stain the second slide that was made and saved from the day before.
3. Refer to the SOP for identifying organisms in CSF; you should be performing the following tests on the specimens indicated.

A-1	Catalase; Bile esculin azide; Optochin
A-2	Oxidase
A-3	No Tests
B-1	Oxidase
B-2	No Tests
B-3	Catalase; Coagulase

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4. Record the results of any biochemical tests that are performed and interpreted. Incubate all susceptibility tests in the CO_2 incubator. Re-incubate any specimen that did not have any growth on any of the three initial plates. Incubate all inoculated tubed media at 37°C, regular oxygen tension. Refrigerate all other P.E. materials.

5. Clean-up as directed.

Third Day

1. Remove biochemical and susceptibility tests from the incubators and primary isolation plates from the storage refrigerator.

2. Interpret the completed biochemical tests and record the results on the last page of this mimeo. Using the SOP for identifying organisms in CSF select the proper name for the organisms isolated.

3. Your results should be as follows:

   A-1 Alpha Streptococcus not group D
   A-2 Presumptive identification Haemophilus sp.
   A-3 No growth

   B-1 Presumptive identification Haemophilus sp.
   B-2 No growth
   B-3 Staphylococcus aureus

4. All of your CSF's should be completely cultured. All culture materials should be discarded and all Petri dishes should be placed in autoclave buckets. Culture tubes should have all tape labels removed and should be consolidated in 90-hole racks in preparation for autoclaving.

5. Clean-up as directed.

CSF Specimen Report Sheet

Spec. no.	Original Gram stain	Colony morphology			Gram stain of growth	Biochemical tests						Conclusion
		Sheep blood agar	Chocolate	MAC		Catalase	Coagulase	Oxidase	BEA	Optochin	Bacitracin	
A-1												
A-2												
A-3												
B-1												
B-2												
B-3												

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY LP 08-280-438
LABORATORY SCIENCE DIVISION 038
MICROBIOLOGY BRANCH

Cerebrospinal Fluid Specimen (CSF) Practical Exercise (6 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Procedures, Basic

PLACE: Classroom

REFERENCES: All previous references

STUDY ASSIGNMENT: None

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: See Annexes A and B

PERSONNEL: Eight instructors, MOS 92B3H

INSTRUCTIONAL AIDS: Chalk, Chalkboard, and eraser.

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Practical exercise

I. INTRODUCTION (5 min)

A. Opening Statement: During this exercise, the remainder of this course, and after being assigned to a clinical laboratory, you will be required to process cerebrospinal fluid specimens (CSF) and to process and identify any organisms that may be present. This exercise is designed to give you practice in performing procedures routinely used in processing CSF's and identifying organisms present.

B. Objective: Given simulated CSF specimens, concentrate and gram stain the sediment; inoculate media and streak for isolation; interpret and report colony morphology; perform and report all tests necessary to identify bacteria present in the specimens, as demonstrated in class.

C. Class Procedure and Lesson Tie-in

1. Application of information received during previous lectures and demonstrations.

2. This exercise using CSF specimens containing various bacteria will be conducted in six hours on three consecutive days; three hours the first day, two hours the second day;

and one hour the third day. Specific tasks assigned by the instructor during each phase will lead to completion of the exercise by the final hour.

II. APPLICATION (235 min)

A. Instructors' Activities

1. Class divided into teams of two students each
2. Equipment and materials issued (see Annexes A and B)
3. Cultures prepared and issued (see Annex C, day prior to PE)
4. Daily instructions given in step-bystep fashion
5. Previous day's activities reviewed
6. Results reviewed with students on a daily basis
7. Questions answered
8. Corrections made
9. Laboratory safety maintained
10. Exercise classroom control

B. Students' Activities

1. Aseptic technique maintained
2. Appropriate media inoculated and streaked for isolation
3. Centrifugation and gram stains performed
4. Colony morphology observed and recorded
5. Biochemical tests performed
6. Culture materials discarded
7. Clean-up as directed
8. Equipment stored properly

C. Organization of Instructional Hours

1. First Day (115 min) (see Annexes A, B, and C)

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a. Selection of cultures by students

NOTE: Each group of two students receives six specimens each labeled A-1 thru A-3 and B-1 thru B-3.

b. Centrifugation of specimens performed

c. Gram stain of sediment performed

d. Inoculation of media performed

e. Cultures incubated

f. Clean-up performed

2. Second Day (80 min)

a. Colony morphology observed and recorded an all specimens

b. Gram stain performed on all growth

c. Perform all appropriate biochemical tests

d. Freshly inoculated media incubated

e. Original plates refrigerated

f. Clean-up performed

3. Third Day (30 min)

a. Biochemical tests interpreted and results recorded

b. Review report sheet for discrepancies

c. All culture materials properly discarded

d. Clean-up performed

III. REVIEW (10 min)

A. Questions from Students

B. Review of Main Points and Work Sheet

1. Colony morphology and gram stain

2. Biochemical test reactions and recording

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3. Proper identification procedures

C. Closing Statement: Background knowledge of the theories and principles of clinical bacteriology, though very important, is insufficient. You must develop the ability to apply that knowledge in an organized and orderly manner so that you can satisfactorily perform your duties a a medical laboratory technician.

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ANNEX A

Tools, Equipment, and Materials

1.	Autoclave, laboratory	1 per class
2.	Refrigerator	1 per class
3.	Incubator	1 per class
4.	Microscope, binocular complete	1 per class
5.	Loop, inoculating	1 per student
6.	Needle, inoculating	1 per student
7.	Forceps, flat tips	1 per student
8.	Marker, wax	1 per student
9.	Glass slides	1 box per student
10.	Disinfectant, germicidal	2 gallons per class
11.	Detergent, surgical	1 gallon per class
12.	Racks, test tube, 90-hole	2 per student
13.	Burner, gas, touchmatic, and tubing	1 per student
14.	Bucket, metal, decontamination	4 per class
15.	Towels, paper	7 pks per class
16.	Immersion oil, microscope	1 per student
17.	Rack, Gram stain	7 per class
18.	Tape, masking	3 rolls per class
19.	Canister, metal, plate	1 per student
20.	Dishwasher	1 per class
21.	Tubes, sterile, 12 x 75 mm	6 per student

ANNEX B

Media and Reagents

1.	Sheep Blood Agar (SBA)	3.5 per student
2.	Pretested plasma, 0.5 ml (coagulase)	0.5 per student
3.	Optochin discs	4 vials per class
4.	Bacitracin discs	4 vials per class
5.	Alcohol bottles (for flaming forceps)	4 bottles per class
6.	30% hydrogen peroxide	4 bottles per class
7.	Oxidase reagent	4 bottles per class
8.	Gram stain reagents (sets)	1 per sink
9.	Sterile saline	5 liters
10.	22% bovine albumin	1 bottle
11.	Chocolate agar	3 per student
12.	MacConkey agar	3 per student

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A. Materials

1. Four bottles of sterile saline for injection (1 liter/bottle)

2. Sheep Blood Agar cultures

   a. *Staphylococcus aureus* - four plates of confluent growth

   b. Alpha hemolytic *Streptococcus* species, not group D - four plates of confluent growth

3. Chocolate agar cultures, four plates of confluent growth of *Haemophilus* species

4. 90-hole test tube racks (one per group of two students)

5. 12 x 75 mm test tubes (sterile), six per student

6. Masking tape (1 roll)

7. Burner with tubing

8. Felt tip marker

9. Sterile swabs and inoculating loop

10. Disinfectant

B. Preparation Procedure

1. Day prior to practical exercise

   a. Inoculate 4 sheep blood agar plates each with *Staphylococcus aureus* and Alpha *Streptococcus*. Inoculate four chocolate agar plates with *Haemophilus* species. Streak all plates for confluent growth.

   b. Incubate all inoculated media in the CO_2 incubator.

2. Day of PE

   a. Add 2 ml of 22% bovine albumin to each liter of sterile saline.

   b. Emulsify the growth on the sheep blood agar plates and the chocolate agar plates; add the emulsified bacteria to the saline; place only one type of bacteria in each bottle of saline.

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c. Dispense 3 ml of the CSF specimen into each of the 12 x 75 mm sterile test tubes for student use.

3. Distribute the CSF specimens according to protocol #1 (one rack of specimens per group of two students)

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PHOTOCOL 1

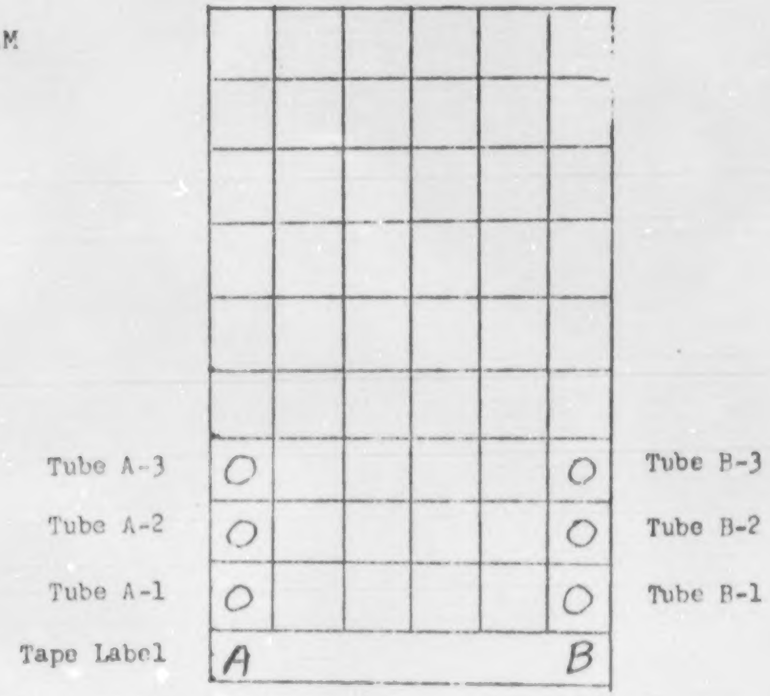
GROUP A

1. Alpha Streptococcus sp.
2. Haemophilus sp.
3. Negative

GROUP B

1. Haemophilus sp.
2. Negative
3. Staphylococcus aureus

DIAGRAM



Tube A-3
Tube A-2
Tube A-1
Tape Label
A
B
Tube B-3
Tube B-2
Tube B-1

NOTE: One rack arranged in this manner is needed for every two students. Care should be taken to arrange organisms exactly as indicated so that they will match the students' instruction sheet (M 08-280-438).

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY LP 08-280-440
LABORATORY SCIENCES DIVISION 038
MICROBIOLOGY BRANCH

API Practical Exercise (2 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory procedures (Basic)

PLACE: Classroom

REFERENCES: API 20 Enterobacteriaceae, Analytab Products, Plainview, New York.

STUDY ASSIGNMENT: Review M 08-280-440-1 prior to class.

STUDENT UNIFORM AND EQUIPMENT: None

TOOLS, EQUIPMENT, AND MATERIALS: See Annex A

PERSONNEL: Eight instructors (MOS 92B3H)

INSTRUCTIONAL AIDS: None

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

METHOD OF INSTRUCTION: Practical Exercise

I. INTRODUCTION (5 min)

A. Opening Statement: With the identification of bacteria becoming more simplified and more efficient, newer and more rapid means of biochemical testing are coming out in the commercial market. The API strip is an example of a rapid, convenient, and fairly accurate method of biochemical testing which outweights conventional biochemicals in many different areas.

B. Objective

Given bacterial specimens, and all necessary materials, prepare, inoculate, and interpret an API 20 Enterobacteriaceae strip, as demonstrated in class.

C. Class Procedure and Lesson Tie-in

1. Application of information presented in demonstration.

2. Familiarization with a different form of biochemical testing.

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II. APPLICATION (60 min)

A. Instructor's Activities

1. First day of PE

a. Equipment issued (see Annex A)

b. Day's activities explained

c. Instructor demonstration of API procedure

d. Laboratory safety maintained

e. Questions answered

f. Corrections of errors made

g. Clean-up supervised

h. Culture material discarded

2. Second day of PE

a. Day's activities explained

b. Laboratory safety maintained

c. Questions answered

d. Correction of errors made

e. Supervise clean-up

f. Culture material discarded

B. Student's Activities

1. First day of PE

a. Area prepared for PE

b. Equipment obtained

c. Make bacterial suspension with designated organism from mixed specimen P.E.

- d. Prepare API strip and incubation tray.
- e. Inoculate strip with bacterial suspension in accordance with M 08-280-440-1
- f. Place trays in non-CO_2 incubator
- g. Equipment stored
- h. Clean-up performed

2. Second day of PE
   - a. Area prepared for PE
   - b. Remove trays from incubator
   - c. Add reagents to appropriate tubes and interpret and record results
   - d. Using API identification book, identify organism
   - e. Show results to instructor
   - f. Equipment stored
   - g. Clean-up performed

III. REVIEW (15 min)

- A. Correct Interpretations Given to Students
- B. Questions from Students
- C. Review of Main Points
  - 1. Preparation of bacterial suspension
  - 2. Preparation and inoculation of API strip
  - 3. Interpretation and recording results
- D. Closing Statement: The API 20 Enterobacteriaceae test strip which you have just used is being accepted and utilized by many laboratories both military and civilian. Therefore, this brief introduction has hopefully prepared you to be aware of other biochemical means of testing.

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038

ANNEX A
INSTRUCTIONAL AIDS

Tools and Equipment

1.	Autoclave, laboratory	1 per class
2.	Incubator, large	1 per class
3.	Pencil, wax	1 per student
4.	Applicator sticks, sterile	1 container per table
5.	Pipets, glass sterile	1 container per table
6.	Bulb, dropper	1 per student
7.	Disinfectant	1 can per table
8.	Saline, tubed sterile	1 per student
9.	Mineral oil, tubed sterile	1 per student
10.	API test strip set, complete	1 per student
11.	Test tube rack, 90-hole	1 per student
12.	Laboratory burner	1 per student
13.	Kovac's reagent	2 bottles per table
14.	Vogues-Proskauer reagents, 1 and 2	2 sets per table
15.	Ferric chloride	2 bottles per table
16.	Water dispenser	3 per table

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY M08-280-440-1
LABORATORY SCIENCE DIVISION
MICROBIOLOGY BRANCH

API Practical Exercise

FIRST DAY

1. Each student will need one API test strip, one complete incubation tray (top and bottom), sterile applicator sticks, one tube of sterile saline, sterile pipets, dropper bulb, and sterile mineral oil.

2. For the cultures to be tested, each student will use an organism designated by the instructor, ie from the mixed specimen PE.

3. Using aseptic technique, remove an applicator stick from the container. With the end of the applicator stick, gently touch the colony to be identified, on the MAC plate. Place the stick into a tube of saline, gently flick the bottom of the tube to provide a swirling motion. Remove the stick and cap the tube. Place used applicator stick into an autoclave bucket.

4. Set up an incubation tray and lid. Record the specimen and student number on the elongated flap of the tray. Dispense about 5 ml of tapwater into the incubation tray to provide a humid atmosphere during incubation. Remove an API strip from the sealed envelope and place it into the incubation tray.

5. To inoculate strips, first remove cap from the tube containing the bacterial suspension and insert a sterile 5 ml Pasteur pipet. Tilt the API tray and fill the tube section of the microtubes by placing the pipet tip against the side of the cupule. Fill both the tube and the cupule section of the CIT, VP, and GEL tubes. After inoculation, completely fill the cupule sections of the ADH, LDC, ODC, and URE tubes with sterile mineral oil.

6. After inoculation, place the plastic lid on the tray and incubate the strips for 18-24 hours at 35-37 C in a non-CO_2 incubator.

7. Clean up as directed.

SECOND DAY

1. Remove strips from incubator, record all reactions not requiring reagents. Interpretation of reactions can be found on the API handout provided.

2. For the addition of reagents, add one drop of 10% ferric chloride to the TDA tube. The reaction should be immediate and can be recorded. To the VP tube add one drop of 40% potassium hydroxide first, then one drop of 6% alpha-napthol. Wait up to 10 minutes before considering the reaction negative. One drop of Kovac's reagent can now be added to the IND tube. The reaction should be read within two minutes.

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3. Identification of the organism is based on a numerical code which is computer formulated. On the small report sheets which are furnished, there are blocks under each biochemical test, both for 24 hours and 48 hours. These blocks are arranged in a series of three with a corresponding single block directly underneath. Each block in the series has a different value for a positive result. Block one is worth 1; block two is worth 2; block three is worth 4 for a total of 7 if all the blocks are positive. If any of the blocks are negative, then a value of zero is given. The following is an example.

	ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND
24H	+	-	+	+	+	+	-	-	+
48H	(1)	(0)	(4)	(1)	(2)	(4)	(0)	(0)	(4)
		5			7			4	

4. When all reactions have been interpreted and recorded, and numerical values given, then identification of the organism is based on the resulting number. Using the API book, either the quick index or the large computer print out book, look up the number to identify the organism.

5. After the organism has been identified then dispose of the strip and incubator tray in the autoclave bucket. Clean up as directed.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-801-1
LABORATORY SCIENCES DIVISION 117
CLINICAL MICROSCOPY BRANCH

Urinalysis Objectives

A. INTRODUCTION

1. Given a urine specimen for microscopic examination, state the best methods of preservation and give reasons for their use.
2. Given a urine specimen for chemical examination, state the best methods for preservation and give reasons for their use.
3. Given a diagram, identify the major anatomical structures of the urinary tract.

B. PHYSICAL PROPERTIES OF URINE

1. Identify the steps involved in performing color, appearance, pH and specific gravity tests on urine.
2. Identify the descriptive terminology of urine production.
3. Given a list of descriptive adjectives, select those that are used in the clinical laboratory to describe urine, color, and appearance.

C. CHEMICAL EXAMINATION OF URINE

1. Identify the steps involved in performing qualitative and/or semi-quantitative tests for protein, reducing sugars, ketones, bilirubin, blood, and nitrites in urine.
2. Given a list of descriptive terms, select those that are used in the clinical laboratory to describe results of chemical determinations on urine.

D. MICROSCOPIC EXAMINATION OF URINARY SEDIMENT

1. List the steps involved in preparing and performing microscopic examination of urinary sediment.
2. Identify blood cells, epithelial cells, casts, crystals, miscellaneous structures and artifacts commonly seen in normal and abnormal urine sediment.

E. URINALYSIS TECHNIQUES

1. Given known urine specimens, detect and identify blood cells, epithelial cells, casts, crystals, miscellaneous structures and artifacts commonly seen in normal and abnormal urine sediment using proper reporting nomenclature as given in class.

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*This mimeo supersedes M 08-280-812-1, 017.

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2. Given known urine specimens, perform qualitative and/or semi-quantitative tests for protein, reducing sugars, ketones, bilirubin, blood, nitrites, color, appearance, pH, and specific gravity along with the proper nomenclature used for reporting the results.

F. GRADED URINALYSIS TECHNIQUES

1. Given known urine specimens, detect and identify blood cells, epithelial cells, casts, crystals, miscellaneous structures, artifacts commonly seen in normal and abnormal urine sediment using proper reporting nomenclature as given in class to a degree of accuracy equivalent to at least 80%.

2. Given known urine specimens, perform qualitative and/or semi-quantitative tests for protein, reducing sugars, ketones, bilirubin, blood nitrates, color and appearance, pH, specific gravity along with proper usage of reporting nomenclature as given in class to a degree of accuracy equivalent to at least 95%.

3. Given known urine specimens, perform appearance, color, pH, and specific gravity testing using proper reporting nomenclature to within a 90% degree of accuracy.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-801
LABORATORY SCIENCES DIVISION 038
CLINICAL MICROSCOPY BRANCH

Introduction to Urinalysis (1 hour)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic)

PLACE: Classroom

REFERENCES:

TM 8-227-6
Lippman, Richard W. Urine and the Urinary Sediment. 2d ed.
Springfield, Illinois: Charles C. Thomas, Published, 1957.
Kark, Robert M., and others. A Primer of Urinalysis. 2d ed.
New York: Harper and Row, Publishers, 1963.

STUDY ASSIGNMENT: After class review M 08-280-801-1 and M 08-280-801-2.

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: M 08-280-801-1 issued at beginning of class.

PERSONNEL: One instructor (MOS 92B2H or 92B3H).

INSTRUCTIONAL AIDS: Chalk, magnetic chalkboard, and eraser; PA system with lapel microphone

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

I. INTRODUCTION (5 min)

A. Opening Statement: Purification of the circulating blood is vitally important for the continuation of life. One of the functions of the circulatory system is the removal of waste products of metabolism from tissue fluid. The blood in turn must be relieved of these substances before thay accumulate in amounts toxic to the body. The urinary system is a group of organs which serves to remove and excrete from the blood those non-volatile waste products which cannot be removed through the respiratory system. The organ which plays the major role in filtration and

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*This lesson plan supersedes LP 08-280-801, 037

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reabsorption is the kidney. During this period of instruction our interest will be directed primarily to this structure in the urinary system.

B. Objectives

1. Given a urine specimen for microscopic examination, state the best methods of preservation and give reasons for their use.

2. Given a urine specimen for chemical examination, state the best methods of preservation and give reasons for their use.

C. Class Procedure and Lesson Tie-in

None

II. EXPLANATION (35 min)

A. Filtration, Reabsorption, and Tubular Secretion of the Nephron

1. Filtration
    a. Glomerulus
    b. Non-selective
    c. Bowman's capsule
2. Reabsorption
    a. Proximal convoluted tubule
    b. Loop of Henle
    c. Distal convoluted tubule
    d. Selective
3. Tubular secretion
    a. Toxic substances
    b. Hydrogen ions
    c. PSP dye

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B. Types of Urine Specimens

1. Qualitative

a. Random

b. First morning void

c. Two hour postprandial

2. Quantitative - 24 hour specimen

C. Special Methods of Collection

1. Catherization

2. Mid-stream clean catch

3. Bladder tap

NOTE: These three are normally bacteria free.

D. Methods of Preservation

1. Refrigeration

NOTE: This is the most commonly used method of preservation.

2. Chemical Preservatives

a. Toluene - Specimen for chemical examination

b. 10% Formalin - Specimen for microscopic examination

E. Questions From Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Filtration, Reabsorption, and Tubular Secretion of Nephron

2. Types of urine specimens

3. Special methods of collection

4. Methods of preservation

B. Closing Statement: No physical examination is complete without a urinalysis. The validity of any urinalysis report is directly proportional to the knowledge, skill, and sense of responsibility of the individual performing the tests. You now have the basis for a better understanding of the physical, chemical, and microscopic tests you will perform during the practical exercises later in this course.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-803
LABORATORY SCIENCES DIVISION 038
CLINICAL MICROSCOPY BRANCH

Physical Properties of Urine (2 hours)

COURSES PRESENTED TO: 311-29B10, Medical Laboratory Specialist (Basic)

PLACE: Classroom and laboratory

REFERENCES:

TM 8-227-6.

Addis, T., and others. Modern Urine Chemistry, August 1974, Ames Company, Chapters IV, V, VI, VIII, XVI.

STUDY ASSIGNMENT: After class read TM 8-227-6, Introduction to Part 3, and Chapter 16, pp 16-1 to 16-3, also, Modern Urine Chemistry, Chapters IV, V, VI, VIII, XVI.

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: M 08-280-803-2 issued at beginning of class.

PERSONNEL: One primary instructor (MOS 92B4H) for 1st and 2d hours.

INSTRUCTIONAL AIDS: Chalk, chalkboard, and eraser; school-prepared urine samples; refractometers; labstixs; litmus paper; liquid color indicators (see Annex A).

TROOP REQUIREMENTS: Not applicable

TRANSPORTATION REQUIREMENTS: Not applicable

TYPE OF INSTRUCTION: Lecture and demonstration (1st and 2d hours).

I. INTRODUCTION (10 min

A. Opening Statement: Blood, the "river of life", can be compared with any geological river, with one very important exception. The usual river flows from its origin to its delta, collecting and carrying pollutants as it journeys to the sea. All its contents, useful and harmful, are eventually deposited irretrievable into the ocean. In a very unique and efficient manner, the human "river of life", by its filtration through the kidneys, discards waste products but returns necessary and useful substances to the body. Because metabolic waste products filtering into the kidneys are constantly changing in relation to body intake and metabolism

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*This lesson plan supersedes LP 08-280-803, 037

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one can expect urine to change in respect to volume, color appearance, specific gravity, and pH. Therefore, the physical examination of this metabolic waste is a great aid in furnishing the medical officer with valuable information regarding the function of the kidneys. Your task is to describe and record accurately physical properties of urine, waste from the "river of life" discarded by the "delta", the kidneys.

B. Objectives

1. List the steps involved in performing color, appearance, pH, and specific gravity tests on urine.

2. List the descriptive terminology of urine production.

3. Given a list of descriptive adjectives select those that are used in the clinical laboratory to describe urine color and appearance.

C. Class Procedure and Lesson Tie-in

There will be an hour practical exercise following this lecture-demonstration.

II. EXPLANATION (60 min)

A. Quantity

1. Normal excretory output of the kidneys

    a. Normally voided

    b. Normal average

2. Factors affecting Normal Urine Production

    a. External temperature

    b. Dietary intake

    c. Seasonal changes

    d. Exercise

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3. Abnormal Urine Production (terms)

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- a. Polyuria
    - (1) Increased urine production
    - (2) Non-pathologic cause
    - (3) Pathologic cause
- b. Oliguria
    - (1) Decreased urine production
    - (2) Non-pathological cause
    - (3) Pathological cause
- c. Anuria
    - (1) Lack of urine production
    - (2) Non-pathologic cause
    - (3) Pathologic cause

4. Quantity determination steps
    - a. Measure sample in graduated cylinder
    - b. Report volume when requested, usually for 24 hour specimen, in MLS.

B. Color

1. Normal
2. Abnormal - other colors
    - a. Red
    - b. Brown
    - c. Black
    - d. Green
    - e. Blue

        f. Orange

        g. Colorless

    3. Color determination steps:

        a. Observe color of fresh specimen

        b. Report as seen

NOTE: Show prepared urine samples which exhibit normal and abnormal coloration.

C. Appearance

    1. Normal

        a. Clear

        b. Hazy

        c. Cloudy (due to excessive concentration of mineral salts)

    2. Abnormal

        a. Cloudy - Pus + Bacteria + WBC's

    3. Appearance determination steps

        (a) Observe fresh, well mixed specimen

        (b) Report as clear, hazy, or cloudy

NOTE: Show prepared specimens of the above.

D. Specific Gravity

    1. Definition: The density of a given sample of urine compared to the density of distilled water (1,000)

    2. Significance

        a. Measure of kidneys' ability to concentrate the urine

        b. Best routine test of total kidney function

QUESTION: Would the specific gravity be less or greater in cases of dehydration or exercise?

ANSWER: The specific gravity would most likely be greater.

3. Method of determination - Refractometer
    a. Principle of refracted light
    b. Temperature correction unnecessary
    c. Light refracted
    d. Simple and rapid
4. Specific gravity determination steps
    a. Clean refractometer
    b. 1 - 2 drops per specimen
    c. View scale
    d. Report normal values 1.003 - 1.030

QUESTION: What is the significance of urine specific gravity?

ANSWER: It is the best routine test of total kidney function.

E. Reaction (pH)
1. The acidity or alkalinity of urine
2. Depends on many factors, values vary widely
3. Methods of determination - pH
    a. Dipstix
    b. pH meter (rare)
    c. Liquid or paper indicators
4. pH determination steps
    a. Liquid universal indicator

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b. Observe color change

c. Compare color (on handout 803-2) to obtain numerical value

d. Report as numerical value, or as state of alkalinity or acidity.

e. Normal values are 4.6 - 8.0.

F. Questions from students

III. SUMMARY (10 min)

A. Review of main points

1. Quantity
2. Color
3. Appearance
4. Specific Gravity
5. pH (Reaction)

B. Closing Statement: As we now see, there is more than meets the eye to the physical properties of urine. Actually, the physical properties are as important to the medical officer as are fingerprints to the criminologist, since both are used in determining an unknown's identity. By accurately performing these tasks, the laboratory technician serves as an important link in the diagnostic chain which expedites patient recovery.

ANNEX A
INSTRUCTIONAL AIDS

SPECIMENS FOR DEMONSTRATION

A. Prepared Urine Specimens

1. Yellow - water food coloring added
2. Straw - water food coloring added
3. Amber - water food coloring added
4. Green - water food coloring added
5. Red - saline red cells added
6. Blue - water food coloring added
7. Brown - water food coloring added
8. Clear - any of above specimen except red.
9. Hazy - addition of insoluble salts to any of the above
10. Cloudy - addition of insoluble salts to any of the above or red specimen.
11. Acid - various amounts of 10% acetic acid to any of the above specimens until desired acid pH is obtained.
12. Alkaline - add various amounts of sodium hydroxide to any of the above specimens until the desired alkaline pH is obtained.

B. Equipment

1. Laboratory Report Slips
2. Refratometer
3. Diastixs
4. Universal Indicator

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SFC Billy W. Martin

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-803-2
LABORATROY SCIENCES DIVISION 037
CLINICAL MICROSCOPY BRANCH

Urinalysis

Color Determination

1. Normal

    a. Yellow

    b. Straw

    c. Amber

Normal colors are due to varying amounts of urochrome.

2. Abnormal

    a. Colorless - absence of urochrome

    b. Red - Blood

    c. Brown - Hemoglobin

    d. Black - Hemoglobin (malaria)

    e. Green - Phenol poisioning (rare)

    f. Blue - Cholera or Typhus (rare)

    g. Orange - medication (Peridium)

Determination of Appearance

1. Clear - no turbidity

2. Hazy - slight turbidity, newsprint may be seen through specimen

3. Cloudy - turbid, newsprint may not be seen through specimen

Determination of Specific Gravity Refractometer

1. Load refractometer

2. Focus with eyepiece

3. Read results on scale 1.000 - 1.035 where shadow crosses scale

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*This mimeo supersedes M 08-280-803-2, 102.

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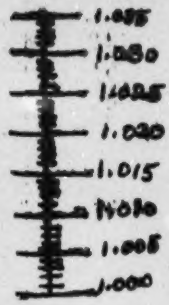
M 08-280-803-2
037


1.035
1.030
1.025
1.020
1.015
1.010
1.005
1.000

Specific Gravity 1.010

Determination of pH Liquid indication

	COLOR	pH
1.	Red	3 or less
2.	Salmon pink	4
3.	Orange	5
4.	Yellow-green	6
5.	Green	7
6.	Blue-green	8
7.	Lavender	9
8.	Purple	10 or more

Report numerically

Add 2 drops of indicator to 0.5 ml of urine and read.

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-806
LABORATORY SCIENCES DIVISION 068
CLINICAL MICROSCOPY BRANCH

Chemical Examination of Urine (3 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic)

PLACE: Classroom

REFERENCES:

Lippman, Richard W. Urine and the Urinary Sediment, 2d ed. Springfield, Illinois: Charles C. Thomas, Publisher, 1967.

Kark, Robert M., and others. A Primer of Urinalysis. 2d ed. New York: Hoeber Division of Harper and Row, Publishers, Inc., 1963.

STUDY ASSIGNMENT: Before class scan TM 8-327-6, Chap 18, pp 18-1 thru 18-14. After class study TM 8-227-6, chap 18, pp 18-1 thru 18-4 and pp 18-9 thru 18-11.

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT AND MATERIALS: M 08-280-806-1 and M 08-280-806-2 issued at beginning of class (one per student)

PERSONNEL: One primary instructor (MOS 92B3H or 92B4H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, and eraser; PA system with lapel mike.

TROOP REQUIREMENTS: Not applicable

TRANSPORTATION REQUIREMENTS: Not applicable

TYPE OF INSTRUCTION: Conference

I. INTRODUCTION (5 min)

A. Opening Statement: Upon initial exposure to the medical laboratory, most inexperienced personnel are somewhat overawed by the complexity of today's advanced technology. However, routine chemical examination of the urine usually does not present a problem because most of the tests involved require only a simple visual interpretation. And yet, these procedures often reveal a great deal regarding the general health of the patient. Because of the relative

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*This lesson plan supersedes LP 08-280-806, 037.

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importance of these screening tests, the need for exacting precision when performing a routine urinalysis is most critical. Carelessness can lead to erroneous results. Since the appearance of abnormal chemical constituents in the urine is frequently indicative of a lurking disease process, the accurate detection of these substances is imperative.

NOTE: Issue M 08-380-806-1 and M 08-280-806-2.

B. Objectives

1. Identify the steps involved in performing qualitative and/or semiquantitative tests for protein, reducing sugars, ketones, bilirubin blood and nitrites in urine.

2. Given a list of descriptive terms, select those that are used in the clinical laboratory to describe results of chemical determinations on urine.

C. Class Procedure and Lesson Tie-in

First three hours of a fourteen hour block of instruction on chemical examination of urine.

NOTE: Use magnetic placards to emphasize main points throughout lecture (see Annex A).

II. EXPLANATION (110 min)

A. Detection of Urinary Proteins

1. Significance of proteinuria
    - a. Functional - no pathological cause (extreme exercise)
    - b. Pathological - result of disease or damage (nephritis)
2. Testing methods (refer to M 08-280-806-1)
    - a. Reagent-impregnated strips (Dipstix)
    - b. Sulfosalicylic acid
    - c. Heat and acetic acid
3. Bence-Jones protein
    - a. Associated disease state - multiple myeloma
    - b. Detection - modified heat and acetic acid method

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(1) Solubility - below 40°C and above 60°C

(2) Insolubility - between 40°C and 60°C

NOTE: At this point, inform the students that most reagent "dipsticks" are impregnated with citrate buffers which maintain the pH of the reaction at 3.0 on the protein test area. This is optimum for the detection of protein when using reagent strips.

QUESTION: What is the difference between functional and pathological proteinuria?

ANSWER: Functional proteinuria has no pathological cause, whereas pathological proteinuria is the result of disease or damage.

B. Detection of Glucose and Other Reducing Substances

1. Presence of glucose in the urine

   a. Diabetes mellitus

   b. Hyperglycemia

2. Presence of other reducing substances in the urine

   a. Galactose - inherited galactosemia

   b. Lactose - pregnancy and lactation

   c. Fructose - metabolic defect (fructosuria)

QUESTION: Large amounts of glucose in the urine are often indicative of what condition?

ANSWER: Diabetes mellitus.

3. Testing methods

   a. Reagent-impregnated strips (Dipstix) - specific for glucose

   b. Clinitest tablets - nonspecific for total reducing substance content

NOTE: Point out that the reagent strips (Dipstix, etc.) are impregnated with the enzyme, glucose oxidase, thus making the test specific for glucose. The Clinitest and Benedict's tests are copper reduction tests, thus making them nonspecific.

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D. Detection of Blood or Hemoglobin

1. Significance of hematuria

   a. Indication of possible urinary tract bleeding

   b. Indication of possible glomerular damage

2. Significance of hemoglobinuria

   a. Elevated serum hemoglobin

   b. Transfusion reactions

   c. Malaria

   d. Extensive burns

3. Testing methods

   a. Reagent-impregnated strips (Dipstix, Hemastix)

   b. Hematest tablets

NOTE: Point out that the above-mentioned tests are more sensitive to hemoglobin than to intact red blood cells.

E. Detection of Bile Pigments

1. Significance of bilirubinuria

   a. Often occurs during jaundice

   b. Possible indication of hepatitis

2. Testing methods

   a. "Shake test" - presumptive screening test

   b. Ictotest

F. Qualitative Test for Urinary Nitrites

1. Indicates bacterial infection of urinary tract

2. Testing method

   a. Reagent-impregnated strips (N-Multistix)

   b. Any pink coloration is positive and suggests 10^5 or more organisms/ml

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NOTE: Instructor may accept questions at any time and may repeat a particular test demonstration upon request.

C. Questions From Students

III. SUMMARY (5 min)

A. Review of Main Points

1. Chemical screening tests

a. Protein

b. Glucose

c. Reducing substances

d. Ketones

e. Blood

f. Bile

g. Nitrites

B. Closing Statement: When the chemical screening tests in a routine urinalysis are performed properly, the laboratory technician has provided the requesting physician with useful and often invaluable information. This clinical data, when reported accurately, gives a good indication of the patient's overall health. Therefore, good technique and accurate reporting must be considered the prime objectives when running a battery of urine chemical screening tests.

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ANNEX A

INSTRUCTIONAL AIDS

Magnetic Placards

1. Chemical Examination of Urine
2. Protein
3. Glucose
4. Ketones
5. Blood
6. Bile Pigments
7. Nitrites

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SFC Billy W. Martin

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *M 08-280-806-2
LABORATORY SCIENCES DIVISION 037
CLINICAL MICROSCOPY BRANCH

Urinalysis

CHEMICAL TESTS FOR GLUCOSE (REDUCING SUBSTANCES)

Dipstix (Ames) and Similar Commercial Products

1. Rapidly dip Dipstix into fresh, well-mixed urine. Remove immediately.
2. Gently tap strip edge against specimen container to remove excess urine.
3. Compare color of test area with glucose color chart.
4. Report as positive or negative.
5. If positive, proceed with the Clinitest.

Clinistix (Ames) and Similar Commercial Products

1. Quickly dip Clinistix into fresh, well-mixed urine. Remove immediately.
2. Compare color of test area with glucose color chart.
3. Report as positive or negative.
4. If positive, proceed with the Clinitest or Benedict's test.

Clinitest (Ames) and Similar Commercial Products

1. Place 5 drops of urine in a 15 by 150 mm test tube and add 10 drops of water.
2. Add one Clinitest tablet.
3. Fifteen seconds after boiling ceases, shake gently, and compare with color chart.
4. Report results according to Clinitest color chart.

NOTE: Extremely high glucose values may form a brown color.

NOTE: This handout should be used in conjunction with appropriate material given in TM 8-227-6. All commercial products have specific instructions for performing test and reporting results.

References:

1. TM 8-227-6, p 18-10, for Benedict's Test.
2. Ames Company, Division of Miles Laboratories, Inc.

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*This mimeo supersedes M 08-280-806-1, 015.

CHEMICAL TESTS FOR ALBUMIN (PROTEIN)

Dipstix (Ames) and Similar Commercial Products

1. Rapidly dip Dipstix into fresh, well-mixed urine. Remove immediately.
2. Gently tap strip edge against specimen container to remove excess urine.
3. Compare color of test area with albumin (protein) color chart.
4. Report Tr, 1+, 2+, 3+, 4+.
5. If positive, proceed with the Sulfosalicylic Acid Method.

Sulfosalicylic Acid Method

1. Place 2 ml of clear urine in a 13 by 100 mm test tube.
2. Add 2 ml of 20 percent sulfosalicylic acid to test tube and mix.
3. Report as follows:

Negative----------clear
Positive----------turbid

NOTE: If urine specimen is cloudy or turbid prior to running test, heat the urine. The turbidity is usually due to inorganic urates and disappears; if it remains, it is due to protein.

References:

1. Ames Company, Division of Miles Laboratories, Inc.
2. Lippman, Richard W. Urine and Urinary Sediment: A Practical Manual and Atlas. 2d ed. il., 1962, page 98.

CHEMICAL TESTS FOR ACETONE (KETONES)

Dipstix (Ames) and Similar Commercial Products

1. Rapidly dip Dipstix into fresh, well-mixed urine. Remove immediately.
2. Gently tap strip edge against specimen container to remove excess urine.
3. Compare color of test area with acetone (keytone) color chart.
4. Report positive or negative.
5. If positive, proceed with the Acetest.

Acetest (Ames) and Similar Commercial Products

1. Place tablet on clean, dry filter paper and add a drop of urine.
2. Compare with Acetest color chart after 30 seconds.
3. Report as follows:

Negative or Positive

References:

1. Ames Company, Division of Miles Laboratories, Inc.
2. Simmons and Gentzkow, p 22.
3. TM 8-227-6, pp 18-3, -4.

CHEMICAL TESTS FOR BLOOD (HEMOGLOBIN)

Dipstix (Ames) and Similar Commercial Products

1. Quickly dip Dipstix into fresh, well-mixed urine. Remove immediately.
2. Gently tap strip edge against specimen container to remove excess urine.
3. Compare color of test area with blood (hemoglobin) color chart.
4. Report positive or negative.

References:

1. Ames Company, Division of Miles Laboratories, Inc.
2. TM 8-227-6, pp 18-3, -4.

CHEMICAL TESTS FOR BILE (BILIRUBIN)

Ictotest (Ames) and Similar Commercial Products

1. Place 5 drops of urine on special mat provided for test.
2. Place tablet in the center of the mat.
3. Flow 2 drops of water over the tablet.
4. Report positive if mat turns purple after 30 seconds, and negative if no color develops after 30 seconds.

References:

1. Ames Company, Division of Miles Laboratories, Inc.
2. TM 8-227-6, p 18-3.

SULKOWITCH TEST (CALCIUM)

1. Place 1 ml of urine in a 15 by 125 mm test tube. If specimen is alkaline, acidify with 10 percent acetic acid (reagent No. 1).
2. Add 1 ml of Sulkowitch's reagent No. 9, mix and allow to stand for 5 minutes.

3. Report as follows:

Decrease - No turbidity
Normal - Hazy
Increase - Turbid

NOTE: This test is a useful, rapid screening method for calcium.

References:

1. TM 8-227-6, p 18-2.
2. TM 8-227-6, p 18-4.

PREPARATION OF REAGENTS USED IN THE CHEMICAL EXAMINATION OF URINE

Reagent No:

1 Acetic Acid, 10 Percent--Place 10 ml of glacial acetic acid into a 100 ml-volumetric flask and dilute to the mark with distilled water.

2 Sulfosalicylic Acid, 20 Percent

Sulfosalicylic acid	20 g
Distilled water	q.s. to 100 ml

Weigh out and transfer the sulfosalicylic acid to a 100 ml volumetric flask. Dissolve in approximately 50 ml of the distilled water and dilute to the mark with distilled water.

3. Sulkowitch's Reagent

Oxalic acid	2.5 g
Ammonium oxalate	2.5 g
Glacial acetic acid	5.0 ml
Distilled water	145.0 ml

Dissolve oxalic acid and ammonium oxalate in distilled water. Add glacial acetic acid and mix well. Transfer to reagent bottle.

References:

1. TM 8-227-6, Attachment 1, REAGENTS.
2. Lippman, Richard W. Urine and Urinary Sediment: A Practical Manual and Atlas. 2d ed. il., 1962, page 98.

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-810
LABORATORY SCIENCES DIVISION 038
CLINICAL MICROSCOPY BRANCH

Microscopic Examination of Urinary Sediment (3 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic)

PLACE: Classroom

REFERENCES:

Lippman, Richard W. Urine and the Urinary Sediment. 2d ed. Springfield, Illinois: Charles C. Thomas, Publisher, 1967.

Kark, R. M., and others. A Primer of Urinalysis. 2d ed. New York: Hoeber Division of Harper and Row, Publishers, Inc., 1963.

STUDY ASSIGNMENT: After class read TM 8-227-6, Chap 17, pp 17-1 thru 17-15, and review M 08-280-810-1.

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: PI 08-280-810-1 issued 5 days prior to class; M 08-280-810-1 issued at start of class.

PERSONNEL: One instructor (MOS 92B3H or 92B4H).

INSTRUCTIONAL AIDS: Chalk, Chalkboard, and eraser; PA system with lapel mike; overhead projector; magnetic placards (see Annex A); S 08-280-810-1 thru S 08-280-810-21 (see Annex B)

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Lecture

I. INTRODUCTION (5 min)

A. Opening Statement: The microscopic analysis of urinary sediment is frequently performed in most clinical laboratories. The importance of this procedure, which has been compared to a biopsy in its significance, cannot be overemphasized. When properly performed and reported, the microscopic examination of urine provides information which is essential to the diagnosis of renal as well as other diseases.

NOTE: At this point issue M 08-280-810-1.

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B. Objectives

1. List the steps involved in preparing specimen and performing microscopic examination of urinary sediment.

2. Given normal and abnormal urine specimens, list the physical characteristics of blood cells, epithelial cells, casts, crystals, miscellaneous structures and artifacts commonly seen in normal and abnormal urine sediment.

C. Class Procedure and Lesson Tie-in

This three-hour lecture and demonstration will be followed by an 18-hour block of practical exercise on the microscopic examination of urine.

II. EXPLANATION/DEMONSTRATION (120 min)

NOTE: Use magnetic placards to emphasize main points throughout lecture (see Annex A).

NOTE: Show 35mm slides of individual microscopic structures at appropriate times throughout lecture (see Annex B).

A. Preparation of Urine Specimen for Microscopic Examination

1. Specimen criteria

a. Fresh

b. Well-mixed

2. Centrifugation

a. Conical centrifuge tube preferred

b. Speed - 1500 RPM

c. Time - 5 minutes

3. Concentration

a. Supernatant used for chemical tests as necessary

b. Stains added to sediment if desired

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4. Preparation of slides
   - a. Limit of two specimens per slide
   - b. Use coverslips for best results

5. Microscopic examination of slides
   - a. Use subdued light
   - b. Screen slide using low power
   - c. Identify objects under high power

B. Organized Sediment

QUESTION: What type of cells may be found in urine?

ANSWER: Red blood cells, white blood cells, epithelial cells.

1. White blood cells
   - a. A few normally found
   - b. Increased in infections and inflammations
   - c. Usually neutrophils
   - d. Identifying characteristics
     - (1) Round or oval
     - (2) Granular cytoplasm
     - (3) Lobulated nucleus (if evident)
     - (4) May contain phagocytized bacteria
   - e. Reported as number per high power field

2. Red blood cells
   - a. Occasionally found in normal state
   - b. Increase may indicate disease or trauma

c. May differ in appearance

(1) Normal

(2) Crenated

(3) Swollen or "ghost"

d. Reported as number per high power field

QUESTION: In what type of urine specimen will red blood cells appear crenated

ANSWER: In a concentrated specimen.

3. Epithelial cells

a. Derived from epithelial tissue lining genitourinary tract

b. Appearance varies according to type

c. Types of epithelial cells

(1) Squamous

(a) Large irregular-edged cells

(b) Small compact nucleus

(2) Transitional

(a) Round to oval

(b) Large nucleus

(3) Renal

(a) Small round to oval cells

(b) Large active nucleus

(c) Differentiated from WBC's by round nucleus

4. Casts

a. Formed in distal convoluted tubules and collecting tubules

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- b. Factors influencing formation
    - (1) Decreased urine flow
    - (2) Low pH
    - (3) Proteinuria
- c. Usually indicative of renal pathology
- d. Identifying characteristics
    - (1) Take shape of tubule of origin
    - (2) Parallel sides
    - (3) Blunt, rounded ends
- e. Variation of types according to composition
    - (1) Hyaline
    - (2) Cellular
        - (a) White blood cell
        - (b) Epithelial
        - (c) Red blood cell
    - (3) Hemoglobin
    - (4) Granular
    - (5) Waxy
    - (6) Fatty
- f. Variations of basic types
- g. Reported as number per low power field

__QUESTION__: When large numbers of casts are observed in the urine microscopic examination, which urine chemical test should you expect to be positive?

__ANSWER__: The test for protein.

5. Pseudocasts
    - a. Cylindroids
    - b. Mucous threads
    - c. Reported as occasional, few, or many

C. Miscellaneous Structures

1. Parasites
    - a. *Trichomonas vaginalis*
    - b. *Schistosoma haemotobium*
    - c. Others
2. Bacteria
    - a. Normally contaminants
    - b. Significant only in fresh specimens
3. Yeasts
    - a. May be contaminants
    - b. Sometimes confused with RBC's
4. Spermatozoa
5. Significant structures reported as occasional, few, or many

D. Artifacts

1. Many contaminants found in urine
2. Usually not reported

QUESTION: Of what importance are artifacts in microscopic analysis of urine

ANSWER: They can cause errors by being identified and reported as significa structures.

E. Unorganized Sediment

1. Normal crystals in acid urine
    - a. Uric acid
        - (1) Yellow or reddish brown
        - (2) Prisms, plates, and/or rosettes
    - b. Amorphous urates
        - (1) Yellow or colorless
        - (2) Granular
        - (3) Heat soluble
    - c. Calcium oxalate
        - (1) Colorless
        - (2) Square with prismatic "X"
    - d. Sodium urate
        - (1) Colorless
        - (2) Elongated plates in fan arrangement
    - e. Calcium sulfate
        - (1) Colorless
        - (2) Elongated plates
2. Normal crystals in alkaline urine
    - a. Amorphous phosphates
        - (1) Colorless
        - (2) Granular
        - (3) Soluble in weak acetic acid
    - b. Triple phosphates

(1) Colorless

(2) Coffin-lid or feathery fan

(3) May appear in slightly acid urine

c. Ammonium urates

(1) Yellow to light brown

(2) Thorn apples, dumbbells, sheaves of fine needles

(3) Precipitate in the presence of free ammonia

d. Calcium carbonates

(1) Colorless

(2) Small dumbbells

e. Calcium phosphates

(1) Colorless

(2) Irregular-edged plates

(3) Found only in alkaline urines

f. Dicalcium phosphates

(1) Colorless

(2) Slender prisms with one pointed end

(3) Rosette or star arrangement

(4) May appear in slightly acid urine

QUESTION: Which test of urine physical properties would assist you most in your identification of urine cyrstals?

ANSWER: Determination of the pH.

3. Abnormal crystals in acid urine

a. Leucine

(1) An amino acid

(2) Indicative of liver pathology

(3) Yellow

(4) Spherical with concentric and radial striations

b. Tyrosine

(1) an amino acid

(2) Indicative of liver pathology

(3) Colorless to yellow

(4) Fine needles in sheaves or rosettes

c. Cystine

(1) An amino acid

(2) Presence due to a metabolic disorder

(3) Colorless

(4) Hexagonal plates

d. Cholesterol

(1) A lipid

(2) Presence due to a metabolic disorder

(3) Rectangular plates with corners missing

4. Sulfa crystals

a. May precipitate after sulfonamide therapy

b. Found only in acid urines

5. All crystals reported as occasional, few, or many

F. Questions From Students

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III. SUMMARY (10 min)

A. Review of Main Points

1. Preparation of urine specimens for microscopic examination.

2. Organized sediment

3. Miscellaneous structures

4. Artifacts

5. Unorganized sediment

B. Closing Statement: You are now familiar with the preparation of urine specimens for microscopic examination. You have learned the proper technique for scanning slides to locate and identify elements of urinary sediment. The characteristics and appearances emphasized in lecture, correlated with the examples shown on slides, have given you information necessary for identifying and reporting microscopic structures encountered in urinary sediment. When performing the microscopic examination of urine, you will often play an important role in contributing to the diagnosis of renal disease.

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ANNEX A
INSTRUCTIONAL AIDS

Magnetic Placards

1. [illegible]CRCSCOPIC EXAM
2. ORGANIZED SEDIMENT
3. LEUKOCYTES
4. ERYTHROCYTES
5. EPITHELIAL CELLS
6. CASTS
7. PSEUDOCASTS
8. MISCELLANEOUS STRUCTURES
9. ARTIFACTS
10. UNORGANIZED SEDIMENT
11. NORMAL CRYSTALS
12. ABNORMAL CRYSTALS
13. SULFA CRYSTALS

ANNEX B
INSTRUCTIONAL AIDS

35 mm Slides

S 08-280-810-1	White Blood Cells
S 08-280-810-2	Red Blood Cells
S 08-280-810-3	Squamous Epithelial Cells
S 08-280-810-4	Transitional Epithelial Cells
S 08-280-810-5	Renal Epithelial Cells
S 08-280-810-6	Hyaline Casts
S 08-280-810-7	WBC Casts
S 08-280-810-8	Epithelial Cell Casts
S 08-280-810-9	RBC Casts
S 08-280-810-10	Granular Casts
S 08-280-810-11	Waxy Casts
S 08-280-810-12	Fatty Casts
S 08-280-810-13	Cylindroids
S 08-280-810-14	Mucous Threads
S 08-280-810-15	*Trichomonas vaginalis*
S 08-280-810-16	*Schistosoma haematobium*
S 08-280-810-17	Yeasts
S 08-280-810-18	Artifacts
S 08-280-810-19	Normal Crystals
S 08-280-810-20	Abnormal Crystals
S 08-280-810-21	Sulfa Crystals

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ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-812
LABORATORY SCIENCES DIVISION 038
CLINICAL MICROSCOPY BRANCH

Urinalysis Techniques (30 hrs)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic) Course

PLACE: Classroom

REFERENCES: All previous references and mimeos

STUDY ASSIGNMENT: Review all previous material

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: Not applicable

PERSONNEL: One primary instructor (MOS 92B4H or 92B3H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, eraser, reagents and equipment (see Annex B)

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Practical Exercise

I. INTRODUCTION (5 min)

A. Opening Statement: While on assignment as a Medical Laboratory Technician, you must be able to perform routine urinalyses with little or no assistance. The results of a properly performed urinalysis are an invaluable aid to the physician.

B. Objectives

1. Given known urine specimens, detect and identify blood cells, epithelial cells, casts, crystals, miscellaneous structures and artifacts commonly seen in normal and abnormal urine sediment using proper reporting nomenclature as given in class.

2. Given known urine specimens, perform qualitative and/or or semi-quantitative tests for protein, reducing sugars, ketones, bilirubin, blood, nitrites, color, appearance, pH, and specific gravity along with the proper nomenclature used for reporting the results.

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*This lesson plan supersedes LP 08-280-812, 037

C. Class Procedure and Lesson Tie-in

Relates to all previous instruction and will be followed by a graded practical exercise.

II. APPLICATION (1,135 min)

A. Instructors' Activities

1. Prepare and issue urine specimens (see Annex A)

2. Issue necessary equipment (see Annex B)

3. Supervise and assist students during P. E.

4. Review students results and counsel when necessary.

B. Students' Activities

1. Perform appropriate test on furnished specimens

a. Chemical

b. Physical

c. Microscopic

2. Record results; to be checked by instructor

3. Clean up and return equipment

III. CRITIQUE (20 min)

A. Questions From Students

B. Review of Main Points

1. Errors in technique

2. Errors in interpretation and reporting nomenclature

C. Closing Statement: Urine contains many constituents, both normal and abnormal. Examinations of urine, when correctly performed and reported, offer the physician important information which will aid him in the diagnosis of the disorder.

LP 08-280-812
038

ANNEX A
INSTRUCTIONAL AIDS

Preparation of Specimens

I. Chemical Examination P.E.

A. Protein - use bovine albumin

Amounts:

1. Trace - 1.0 ml/900 ml H_2O
2. 1+ - 2.0 ml/900 ml H_2O
3. 2+ - 4.0 ml/900 ml H_2O
4. 3+ - 10.0 ml/900 ml H_2O
5. 4+ - 15.0 ml/900 ml H_2O

B. Glucose - use dextrose (Clinitest Rx)

Amounts:

1. Trace - 2.5 ml/900 ml H_2O
2. 1+ - 3.0 ml/900 ml H_2O
3. 2+ - 5.0 ml/900 ml H_2O
4. 3+ - 9.0 ml/900 ml H_2O
5. 4+ - 16.0 ml/900 ml H_2O

C. Ketones - use acetone (Dipstick Rx)

Amounts:

1. Small - 1.5 ml/900 ml H_2O
2. Medium - 2.5 ml/900 ml H_2O
3. Large - 3.5 ml/900 ml H_2O

D. Blood - use Hemolyzed blood

Amount: 2 drops with "dispo" pipette/900 ml H_2O

E. Bilirubin - use Ferric Chloride

Amount: 1.5 ml/900 ml distilled H_2O

NOTE: Do not use calcium in any specimen containing Ferric Chloride; calcium will precipitate out.

F. Calcium - use calcium chloride

Amounts:

1. Increased - 10.0 ml/900 H_2O
2. Normal - 1.0 ml/900 H_2O
3. Decreased - 900 ml distilled H_2O

NOTE: Orange G or food coloring is added to specimens to produce a realistic color. Prepare 10 specimens in sufficient quantity so that each student will receive 5.0 ml of each specimen.

II. Physical Properties P.E.

A. Color

1. Red - use RBC's
2. Colorless - use H_2O
3. Yellow - use food coloring
4. Straw " " "
5. Amber " " "
6. Green " " "
7. Brown " " "
8. Orange " " "

B. Appearance

1. Clear - no addition of non-dairy creamer to specimen
2. Hazy - add non-dairy creamer to specimen
3. Cloudy - add non-dairy creamer to specimen

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C. Specific Gravity

Use varying amounts of NaCl in specimens.

D. Reaction (pH)

1. Use acetic acid to obtain acid pH.
2. Use Sodium Hydroxide to obtain Alkaline pH.

NOTE: Class will be divided into equal groups of students. Prepare 10 specimens in sufficient quantity so that each group will receive 100 ml of each specimen in a urine collection cup.

III. Microscopic Examination P.E.

A. Show 35 mm slides, to include organized sediment, unorganized sediment, miscellaneous structures and artifacts. Use slides listed in Annex B, LP 08-280-810.

B. Specimens for wet-mounts obtained from hospital. Specimens are screened for microscopic contents.

NOTE: Students should be exposed to both normal and abnormal urine specimens. Number of specimens depends upon availability.

ANNEX B
INSTRUCTIONAL AIDS

Equipment and Reagents

I. Chemical Examination P.E.

A. For practice P.E., each student will receive a kit containing each of the following items:

1. Labstix - 1 bottle
2. Acetest tablets - 1 bottle
3. Clinitest tablets - 1 bottle
4. Ictotest - 1 box
5. Disposable pipettes - 14
6. Test tube rack (49 hole - 1
7. 14 X 100 mm test tubes - 30
8. 15 X 125 mm test tubes - 10
9. Laboratory report sheets - M 08-280-808-2

NOTE: Number of kits required: 120

B. For practice P.E., every two students will receive each of the following items:

1. Sulfosalicylic Acid - 1 bottle
2. Sulkowitch Reagent - 1 bottle
3. Distilled H_2O - 1 bottle
4. Circular Filter Paper - 1 box

II. Physical Properties P.E.

A. For practice P.E., class will be divided into eight (8) groups.

B. Each group will receive the following items:

1. Laboratory Report Sheets - M [illegible]0-805-2
2. Refractometer - 1

3. Universal indicator - 1 bottle
4. Disposable pipettes - 10
5. Twenty hole rack - 1
6. 10 X 75 mm test tubes - 10

III. Microscopic Examination P.E.

A. Students will be shown 35 mm slides, to include organized sediment, unorganized sediment, miscellaneous structures and artifacts.

B. For practice wet-mount P.E., each student will receive:

1. Microscope
2. Coverslips
3. Glass slides
4. Laboratory report sheets - M 08-280-812-2

ACADEMY OF HEALTH SCIENCES, UNITED STATES ARMY *LP 08-280-813
LABORATORY SCIENCES DIVISION 038
CLINICAL MICROSCOPY BRANCH

Urinalysis Techniques (Graded) (8 hours)

COURSES PRESENTED TO: 311-92B10, Medical Laboratory Specialist (Basic) Course

PLACE: Laboratory

REFERENCES: All previous references and mimeos

STUDY ASSIGNMENT: Review all previous material

STUDENT UNIFORM AND EQUIPMENT: Not applicable

TOOLS, EQUIPMENT, AND MATERIALS: None

PERSONNEL: One primary instructor (MOS 92B3H or 92B4H)

INSTRUCTIONAL AIDS: Chalk, chalkboard, eraser, reagents and equipment (see Annex B)

TROOP REQUIREMENTS: None

TRANSPORTATION REQUIREMENTS: None

TYPE OF INSTRUCTION: Practical exercise

I. INTRODUCTION (10 min)

A. Opening Statement: You have completed the didactic and practical phases of the course. This graded practical exercise will help to determine your proficiency in performing routine tests and examinations of urine.

B. Objectives

1. Given known urine specimens, detect and identify blood cells, epithelial cells, casts, crystals, miscellaneous structures, artifacts commonly seen in normal and abnormal urine sediment using proper reporting nomenclature as given in class to a degree of accuracy equivalent to at least 80%.

2. Given known urine specimens, perform qualitative and/or semi-quantitative tests for protein, reducing sugars, ketones, bilirubin, blood nitrates, color and appearance, pH, specific

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*This lesson plan supersedes LP 08-280-813, 037

gravity along with proper usage of reporting nomenclature as given in class to a degree of accuracy equivalent to at least 95%.

3. Given known urine specimens, perform appearance, color, pH, and specific gravity testing using proper reporting nomenclature to within a 90% degree of accuracy.

4. Given urine specimens, detect and identify structures commonly seen in normal and abnormal urine sediment [illegible] include, organized sediment, unorganized sediment. Miscellaneous structures, and artifacts along with proper usage of reporting nomenclature with a degree of accuracy equivalent to 80%.

C. Class Procedure and Lesson Tie-in

Relates to all previous material.

II. APPLICATION (450 min)

A. Instructors' Activities

1. Prepare and issue keyed specimens (see Annex A)

2. Issue necessary equipment (see Annex B)

3. Supervise graded P. E.

4. Grade students' results (see Annex C)

B. Students' Activities

1. Perform appropriate tests on furnished specimens

   a. Chemical

   b. Physical

   c. Microscopic

2. Record results; turn in to instructor

3. Clean up and return equipment

III. SUMMARY (10 min)

A. Question From Students

B. Review of Main Points

1. Errors in technique

2. Errors in interpretation and reporting nomenclature

C. Closing Statement: This graded practical exercise has been designed to point out your deficiencies in the procedures performed. Any deficiencies you experienced should be examined as soon as possible.

ANNEX A
INSTRUCTIONAL AIDS

Preparation of Specimens

I. Chemical Examination P.E.

A. Protein - use Bovine Albumin

Amounts:

1. Trace - 1.0 ml/900 ml H_2O
2. 1+ - 2.0 ml/900 ml H_2O
3. 2+ - 4.0 ml/900 ml H_2O
4. 3+ - 10.0 ml/900 ml H_2O
5. 4+ - 15.0 ml/900 ml H_2O

B. Glucose - use Dextrose (Clinitest Rx)

Amounts:

1. Trace - 2.5 ml/900 ml H_2O
2. 1+ - 3.0 ml/900 ml H_2O
3. 2+ - 5.0 ml/900 ml H_2O
4. 3+ - 9.0 ml/900 ml H_2O
5. 4+ - 16.0 ml/900 ml H_2O

C. Ketones - use Acetone (Dipstick Rx)

Amounts:

1. Small 1.5 ml/900 ml H_2O
2. Medium - 2.5 ml/900 ml H_2O
3. Large - 3.5 ml/900 ml H_2O

D. Blood - use hemolyzed blood

Amount: 2 drops with "dispo" pipette/900 ml H_2O

E. Bilrubin - use Ferric Chloride

Amount: 1.5 ml/900 ml distilled H_2O

NOTE: Do not use Calcium in any specimen containing Ferric Chloride; Calcium will precipitate out.

F. Calcium - use Calcium Chloride

Amounts:

1. Increased - 10.0 ml/900 H_2O
2. Normal - 1.0 ml/900 H_2O
3. Decreased - 900 ml distilled H_2O

G. Nitrite - use Sodium Nitrite

Amount: 4.0 ml/900 H_2O

NOTE: Orange G or food coloring is added to specimens to produce a realistic color. Prepare 10 specimens in sufficient quantity so that each student will receive 5.0 ml of each specimen.

II. Physical Properties P. E.

A. Color

1. Red - use RBC's
2. Colorless - use H_2O
3. Yellow - use food coloring
4. Straw " " "
5. Amber " " "
6. Green " "
7. Brown " " "
8. Orange " " "

B. Appearance

1. Clear - no addition of non-dairy creamer to specimen
2. Hazy - add non-dairy creamer to specimen
3. Cloudy - add non-dairy creamer to specimen

C. Specific Gravity

Use varying amounts of NaCl in specimens.

D. Reaction (pH)

1. Use acetic acid to obtain acid pH.

NOTE: Classroom will be divided into 8 stations. Prepare 10 specimens in sufficient quantity so that each station will receive 100 ml of each specimen in a urine collection cup. One student at a time will perform the necessary tests at the assigned station.

III. Microscopic Examination P.E.

A. Show 35 mm slides, to include organized sediment, unorganized sediment, miscellaneous structures and artifacts. Use slides listed in Annex B, LP 08-280-810.

B. Specimens for wet-mounts obtained from hospital. Specimens are screened for microscopic contents.

NOTE: Students should be exposed to both normal and abnormal urine specimens. Number of specimens depends upon availability.

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ANNEX B
INSTRUCTIONAL AIDS

Equipment and Reagents

I. Chemical Examination P. E.

A. For graded P.E., each student will receive a kit containing each of the following items:

1. Labstix - 1 bottle
2. Acetest tablets - 1 bottle
3. Clinitest tablets - 1 bottle
4. Ictotest - 1 box
5. Disposable pipettes - 14
6. Test tube rack (40 hole) - 1
7. 14 X 100 mm test tubes - 20
8. 15 X 125 mm test tubes - 10
9. Laboratory report sheets - M 08-280-808-2

NOTE: Number of kits required: 120

B. For graded P.E., every two students will receive each of the following items:

1. Sulfosalicylic acid - 1 bottle
2. Distilled H_2O - 1 bottle
3. Circular filter paper - 1 box

II. Physical Properties P. E.

A. For graded P.E., classroom will be divided into eight (8) stations.

B. Each station will receive the following items:

1. Laboratory report sheets - M 08-280-805-2
2. Refractometer - 1

3. Universal indicator - 1 bottle
4. Disposable pipettes - 10
5. Twenty hole rack - 1
6. 10 X 75 mm test tubes - 10

III. Microscopic Examination P. E.

A. Students will be shown 35 mm slides, to include organized sediment, unorganized sediment, miscellaneous, structures and artifacts.

B. For grade wet-moun[illegible].E., each student will receive:

1. Microscope
2. Coverslips
3. Glass slides
4. Laboratory report sheets - M 08-280-812-2

ANNEX C
INSTRUCTIONAL AIDS

LP 08-280-813
038

Grading System

MAX P. E. COURSE POINTS - 70 PTS

I. Chemical G.P.E.

#CORRECT RESPONSES	COURSE POINTS
100	20
99	19.5
98	18.5
97	18
96	17
95	16
94	FAIL

II. Physical Properties G.P.E.

#CORRECT RESPONSES	COURSE POINTS
40	10
39	9.5
38	9
37	8.5
36	8
35	FAIL

III. Microscopic Exam of Uninary Sediment

#CORRECT RESPONSES	COURSE POINTS
25	40
24	39
23	37
22	34
21	31
20	28
19	FAIL

NOTE: PAGE NO. 1256 IS MISSING; HOWEVER, ALL MATERIAL IS INCLUDED.

Chapter 1

INTRODUCTION TO BACTERIOLOGY

Bacteria are microscopic, unicellular organisms of the plant kingdom, reproducing by binary fission. The taxonomic position of bacteria in the plant kingdom is given below.

TAXONOMIC POSITION

Kingdom Plant

- Subkingdom Thallophyta (not forming embroys)
  - Phylum Schizomycophyta (noncholorophyll producing)
    - Class Schizomycetes (multiplying by binary fission)
      - Order Eubacteriales (nonbranching, true bacteria)
      - Order Actinomycetales (usually elongated and branching bacteria)
      - Order Chlamydobacteriales (filamentous, algalike water forms)
      - Order Myxobacteriales (slime bacteria)
      - Order Spirochaetales (protozoanlike flexible spirals)

The nature of the species found in each of the five orders of bacteria directs the investigation of medical bacteriology to the Orders Eubacteriales, Actinomycetales, and Spirochaetales. The Order Eubacteriales contains the majority of those bacterial species exhibiting pathogenicity for humans.

CLASSIFICATION

One set of characteristics cannot be used to differentiate all pathogenic bacteria. Classification must be based on a multitude of characteristics contained in the three categories of morphological characteristics, physiological properties, and other factors.

Classification According to Morphological Characteristics

Morphological characteristics include size, shape and arrangement of cells, and internal cellular structures. The three principal shapes of bacteria and their average sizes are given in table 1-1. These forms vary considerably in size, and their unit of measurement is the micron or μ. $1\mu = 1/1,000$ mm. or 1/25,000 inch.

Table 1–1. Morphological Characteristics.

Shape	*Terminology (singular, plural)*	*Average Size (microns)*
spherical	coccus, cocci	1.0μ
rod-shaped	bacillus, bacilli	$0.5\mu \times 2.0\mu$
spiral-shaped	spirillum, spirilla.	$0.2\mu \times 10\mu$

(coccobacillary forms are intermediate between spheres and rods)

Cellular arrangement is the result of the type of cellular division. See table 1-2.

Table 1-2. *Arrangement of Bacterial Cells.*

Arrangement	*Terminology*	*Type of Division*
COCCUS (See fig. 1-1).		
single (A)	coccus	------
pair (B)	diplococcus	in one plane
chain (C)	streptococcus	in one plane
cluster (D)	staphylococcus	in many planes
four (E)	tetrad (*gaffkya*).	two or more planes
eight (E)	cube (*sarcina*).	two or more planes
BACILLUS (See fig. 1-2).		
single (A)	bacillus	------
pair (B)	diplobacillus	in one plane
chain (C)	streptobacillus	in one plane
palisade (D)	palisade	in one plane (post-fission whipping)
SPIRILLUM, always occur singly		
COCCOBACILLUS, same as for coccus		

Bacteria possess complex cell structures which may be categorized as general cell structures (possessed by all bacteria) and special cell structures (possessed in various combinations by some bacteria). See table 1-3.

Simplicity of cell structure make it impossible to classify bacteria on the basis of morphology alone. Physiological properties are added to take advantage of the variety of nutrients utilized by bacteria and their manner of metabolism.

Classification According to Physiological Properties

Classification according to physiological properties is based on growth requirements —primarily the need for an inorganic or an organic source of carbon.

Autotrophic bacteria obtain energy and grow on inorganic media, employing CO_2 as

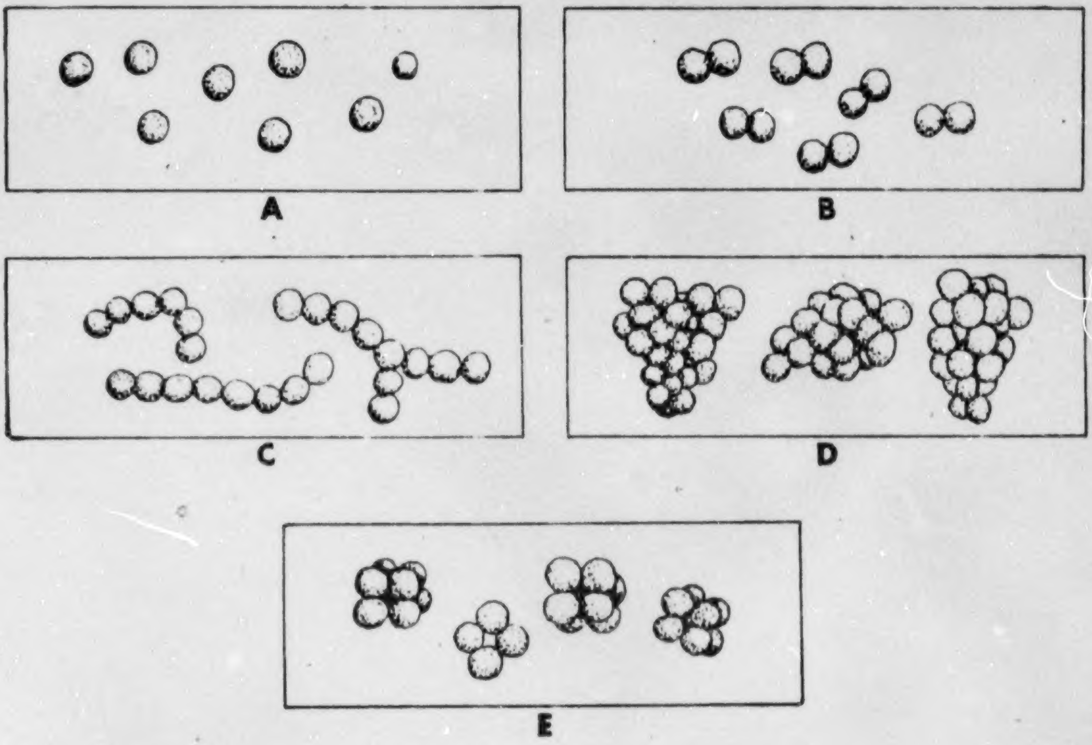

Figure 1-1. *Arrangement of Cocci.*

Table 1–3.

GENERAL CELL STRUCTURES

Cell-wall—a thin, rigid cellulose covering that encloses the protoplasm of the cell, giving rigidity to the bacterial shape.

Cytoplasmic membrane—a semipermeable membrane located directly beneath the cell wall, governing osmotic activity.

Cytoplasm—the protoplasmic or vital colloidal material of a cell exclusive of that of the nucleus.

Nucleus—diffused chromatin material responsible for replication of the cell.

SPECIAL CELL STRUCTURES

Capsule—an accumulation of high molecular weight, excretory substances (slime layer) around a bacterium or bacteria. A capsule serves as defense against phagocytosis.

Flagellum—a protoplasmic strand of elastic protein originating in the cytoplasm and extending from the body of the cell. A flagellum serves as an organ of locomotion. The arrangement of flagella (pleural) is peculiar to the species.

Spore—metabolically inactive resistant bodies formed by a vegetative bacterium to withstand an unfavorable environment. Only bacilli form spores. The position and sizes of the spore within a bacillus is peculiar to the species.

Inclusion bodies—vacuoles of reserve or waste materials contained within the cytoplasm.

their sole source of carbon. Autotrophs begin with CO_2 and NH_3 and from these build an entire protoplasmic structure of protein, fat and carbohydrates, using the oxidation of ammonia to obtain energy for their other processes.

Heterotrophic bacteria obtain energy from organic carbon sources. Heterotrophs require the addition of sugars, amino acids, purines, pyrimidines, and vitamins to their culture media, using the fermentation of sugar as the primary source of energy. *Parasitic bacteria* are heterotrophs. They have become adapted to an environment in which many kinds of organic materials are normally available. In many cases such organisms have lost their ability to synthesize certain complex organic substances needed for their growth. Bacterial parasites require living organic matter for nutrients and may cause harm to the host. *Saprophytic bacteria* are heterotrophs which utilize decaying organic matter for nutrients and usually do not harm the host.

By preparing artificial media containing various growth requirements, bacteriologists are able to study the growth characteristics of certain bacteria. Growth requirements of bacteria associated with a parasitic or saprophytic existence in man include optimum temperature, oxygen, pH, and suitable nutri-

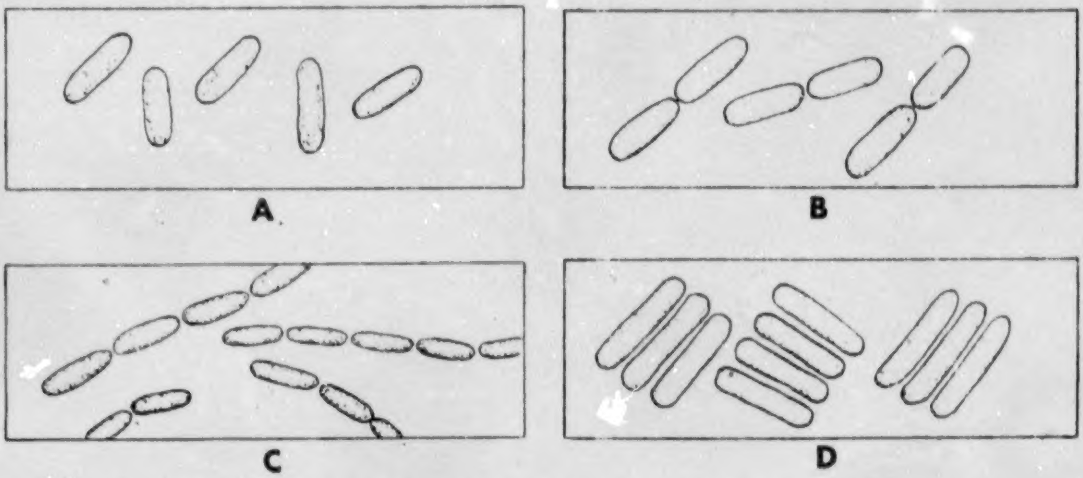

Figure 1–2. Arrangement of Bacilli.

ents. These growth factors are discussed in chapter 4.

Classification According to Other Factors

Classification of bacteria according to pathogenic responses and immunology will be discussed with each species or genus, along with the laboratory techniques for cultivation, isolation and identification of pathogenic and saprophytic species pertinent to humans.

REPRODUCTION OF BACTERIA

Bacteria are replicating or self-duplicating systems. Bacterial cells grow increasing protoplasm by a series of interdependent, complex reactions. Cell growth results in elongation or enlargement, increase in cytoplasm and formation of nuclear material sufficient to serve two cells. Each new cell formed is potentially capable of dividing into daughter cells. One cell deposited on a suitable solid medium, in an appropriate environment for growth, is capable of producing its progeny in the form of a clone or isolated bacterial colony. Bacteria reproduce by splitting at right angles to their longest axis —a process called binary fission. The generation time—or time elapsing between formation of a cell and its division—is dependent upon many factors including the medium, temperature, age of culture and the bacterial strain, species, or genus. The average generation time is 15-20 minutes for enteric bacilli and several hours for the Tubercle bacilli.

The typical growth curve of a bacterial culture exhibits four phases—the lag phase, the logarithmic growth phase, the stationary phase, and the senescent phase (see fig. 1-3). The *lag phase* (A) commences immediately upon inoculation of bacteria to media. In the lag phase a number of chemical changes are taking place within most of the cells, despite the absence of cell division. The average cell size increases as much as 2-3 times above that of the original size. Reproduction begins toward the end of the lag phase. The *logarithmic growth phase* (B) exhibits rapid growth and constant generation time determined by the interplay of inherited characteristics of the organism and the environmental conditions. The reproduction rate begins to slow down in the *stationary phase* (C). The generation time increases and finally the number of viable bacteria become stabilized. In the *senescent phase* (D) the environmental resistance begins to overpower the reproductive capacity of the bacteria. A progressively decreasing rate of multiplication is observed during this period. The number of viable bacteria decrease slowly at first, then rapidly until a constant death rate is maintained. Growth, multiplication, and death of bacteria must be considered as highly dynamic processes involving numerous reactions within the cell and between it and its environment. The first two phenomena represent the establishment of dynamic equilibria which, when fully established, culminate in the steady state noted during the logarithmic phase of multiplication. Any change in cell or medium results in an alteration of the steady-state conditions; and, if the cell is unable to adjust to change, cellular activity becomes inhibited.

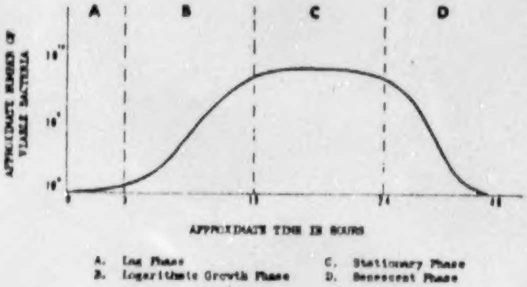

Figure 1-3. Typical Growth Curve of a Bacterial Culture.

RELATIONSHIP OF BACTERIA TO ENVIRONMENT

The interresponse between bacteria and their environment can be manifested three ways: (1) the influence of the environment upon the bacterial cell, (2) the influence of the cell upon its environment, and (3) the interresponse of bacterial organisms within the same environment.

By varying the constituents of media and atmospheric conditions, one can effect environmental influence upon bacteria. The more adaptable an organism is able to be in its utilization of nutrients, the wider is its range of possible environments, and the greater its chance of survival. Variations from the previous "norm" can come about through adaption or mutation, or both. Pathogenic bacteria lose their virulence and capsules (if encapsulated) when cultivated on artificial media, but regain both on passage through animals.

The influence of the cell upon its environment (physiological) may be exhibited in pathogenicity. It is postulated that parasites orginated from saprophytic ancestors which became adapted to growth with or upon other species and finally to growth within living tissue. Parasites eliciting disturbing reactions in a host are called pathogens. Virulence is employed as a measure of the degree of pathogenicity of a bacterium (through invasiveness, production of toxins, or combination of these). Virulence is not a property of the bacterium alone, but is associated with the nature of the host and with the route of introduction into the host. Virulence is determined by whatever means a particular bacterium has for gaining entrance into living tissue; and once it is in, the means it has for combating various bacteriostatic or bactericidal properties of the host. Saprophytic forms are quickly removed through phagocytosis. Pathogenic forms are more resistant to phagocytes and other forces and thereby cause inflammatory responses.

Least is known concerning the interresponse of bacterial organisms within the same environment—the "normal flora" situation. Rarely do pure cultures exist in nature. Mixed flora of any population is the product of not only the physical and chemical environment on each individual group of organisms of that flora, but also of the biological environment.

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Chapter 4

CULTIVATION OF BACTERIA

Bacteria generally cannot be identified on the basis of their microscopic morphology and staining reactions. It is usually necessary to inoculate samples of the specimen to artificial culture media (solid or liquid) and perform studies on growth obtained following media incubation. This necessitates an understanding of the growth requirements for cultivation of bacteria.

GENERAL CONSIDERATIONS

Any material which will support the growth and reproduction of bacteria may be termed a culture medium. Culture media provides an artificial environment for the laboratory study of bacteria. The development of microorganisms on or in an artificial medium is dependent upon the following factors:

1. Presence of suitable nutrients and other elements necessary for synthesis of protoplasm.
2. Proper gaseous environment. This may consist of normal atmosphere, increased carbon dioxide, or complete lack of atmospheric oxygen.
3. Appropriate pH of the medium.
4. Presence of an adequate amount of moisture.
5. Proper treatment of incubation.

Bacteria vary considerably with respect to the above requirements. The problem confronting the clinical bacteriologist is that of selecting the most suitable environment for propagation of any disease-producing organisms present in specimens. If the cultural criteria are met, adequate growth should occur. The cultural environment for disease-producing bacteria is also ideal for many of the naturally occurring saprophytic organisms. Saprophytic organisms interfere with the growth of possible pathogens. Aseptic technique should be utilized to prevent contaminants from being introduced during procedures.

FACTORS AFFECTING BACTERIAL GROWTH

Temperature

Bacteria vary considerably with respect to temperature requirements. *Psychrophilic* forms grow more readily at lower temperatures (15°-26° C.); *mesophilic* forms grow best at 30°-37° C.; *thermophilic* forms grow best at 50°-60° C. The majority of human pathogens are mesophilic, growing best at 37° C.

A constant temperature incubator adjusted to 37° C. and containing sufficient moisture satisfies the temperature requirement.

Gaseous Environment

It is essential that the proper gaseous environment be furnished when attempting to cultivate bacteria. The specific needs apply to oxygen and carbon dioxide. Oftentimes, the failure to isolate pathogenic microorganisms from clinical materials is due to inadequate provisions with respect to aeration.

OXYGEN REQUIREMENTS

Bacteria are divisible into two broad groups on the basis of their oxygen requirements: (1) aerobic forms which require free oxygen from growth, and (2) anaerobic forms which require the absence of free oxygen for growth. Aerobic and anaerobic bacteria are further divided into several categories, defined below.

Obligate Aerobes. An obligate aerobe *must* have free atmospheric oxygen in order to grow. These forms grow best on agar media in a normal atmosphere. They will also grow in the upper portion of a broth medium. Some members of the genus *Bacillus* are strict aerobes.

Microaerophils. These forms require a greatly reduced atmosphere of oxygen for optimal growth such as that supplied in the lower portion of a broth medium. The organisms will also grow well on an agar plate, when incubated in an atmosphere of increased carbon dioxide (candle jar). *Hemophilus spp.* and many streptococci are microaerophils.

Facultative Organisms. Those organisms capable of adapting to either presence or absence of atmospheric oxygen fall into this group.

Obligate Anaerobes. These forms require the strict absence of atmospheric oxygen for growth. Free oxygen is toxic to obligate anaerobes as a result of producing enzyme destruction or inactivation. Special measures must be taken to remove and exclude oxygen from the cultures during incubation. Some pathogenic bacteria are obligate anaerobes on primary isolation, yet will adapt to aerobic conditions upon subculture. Examples of true obligate anaerobes are represented in the genus *Clostridium* and some streptococci.

ANAEROBIC METHODS

Although many methods for preparing anaerobic cultures have been devised, the procedures listed below have been widely used with considerable success:

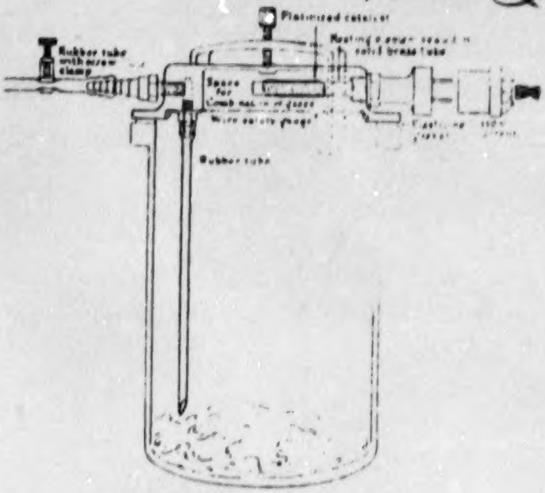

Figure 4–1. Brewer Anaerobic Jar

Brewer Anaerobic Jar Technique. The Brewer anaerobic jar (fig. 4-1) is a large cylindrical vessel which will accommodate several agar plates and test tube cultures.

The complete assembly for operating the Brewer jar in securing anaerobiasis is shown in figure 4-2. Either hydrogen or illuminating gas may be employed.

Since illuminating gas is readily available, it is far more practical and less dangerous to use in securing anaerobiasis with a Brewer jar. If illuminating gas is employed, the following stepwise procedure is used:

1. After placing the inoculated media in the jar, rim the jar top with sealing compound. A thin coating of "Cello-Seal" or one of the newer silicone sealing prepartions is recommended. If "Plasticine" is used for the first time, a coat of vaseline should be applied to the rim of the vessel before putting the "Plasticine" in place.
2. Secure the metal top of the jar by tightening the U clamp snugly (do not overtighten).
3. Turn the three-way cock to vacuum and evacuate until there is a difference of 20 centimeters between the two mercury columns of the manometer. The vacuum source may be supplied by a Richard's pump or the standard electrically powered vacuum pump.
4. When 20 centimeters of displacement is obtained, turn the three-way cock to the gas supply and allow gas to flow into the jar. Disconnect the vacuum pump.

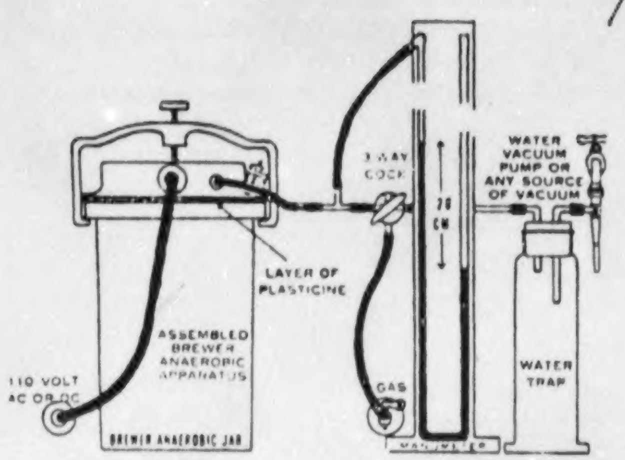
3 WAY COCK
WATER VACUUM PUMP OR ANY SOURCE OF VACUUM
76 CM
LAYER OF PLASTICINE
ASSEMBLED BREWER ANAEROBIC APPARATUS
110 VOLT AC OR DC
GAS
WATER TRAP
BREWER ANAEROBIC JAR
MANOMETER

Figure 4–2. Brewer Anaerobic Jar Assembly with Electrical Outlet, Manometer, and Vacuum Source.

5. **Maintain the gas supply and electrical connection for a 30–45 minute period.**
6. **Disconnect the electric cord from the heating element. Turn off the gas supply by tightening the clamp on the rubber tubing at the jar lid. Turn off the gas supply at its source.**
7. **Disengage the rubber tubing from the manometer and place the jar in an incubator.**

When operating a Brewer jar with illuminating gas, a tube of soda lime is placed inside the jar along with the cultures. The soda lime will absorb the excess carbon dioxide formed and thereby prevent a lowering of pH in the culture media.

During the process of obtaining anaerobiasis, proper functioning of the apparatus is evidenced by the formation of droplets of water on the inside of the jar.

Thioglycollate broth. This medium is excellent for cultivating strict anaerobes as well as aerobes. The sodium thioglycollate in the medium acts as a reducing agent and good anaerobiasis is produced in all but the very upper portion of the medium. Thioglycollate fluid medium is widely used as a routine broth medium for most bacteriological analyses.

CARBON DIOXIDE REQUIREMENTS

Many microorganisms require a carbon dioxide concentration above normal atmospheric levels. An atmosphere containing 2–3 percent carbon dioxide is adequately supplied by incubating cultures in a candle jar. See figure 4–3. Any widemouthed vessel with an air-

Figure 4–3. Candle Jar for Securing a Cultural Atmosphere of Increased Carbon Dioxide.

tight lid will suffice, provided it is large enough to accommodate several cultures. Inoculated tubes or plates of media are placed in the vessel. A lighted candle is then placed in the container above the cultures. After securely tightening the lid, the burning candle will be extinguished when about 2–3 percent carbon dioxide has accumulated. The candle should be placed as high as possible in the candle jar to permit accumulation of CO_2 which extinguishes the flame. When an atmosphere of 10 percent carbon dioxide is needed the following method may be employed.

1. Place the inoculated media inverted in a vessel equipped with an airtight lid.
2. Add 1 gm of sodium carbonate for each 2.5 liter of vessel capacity, to a small open container.
3. Place the sodium carbonate container inside the vessel with the cultures.
4. Cover the sodium carbonate with cotton and add 100 ml of 3 percent sulfuric acid for each gram of sodium carbonate used.
5. Quickly secure the lid on the carbon dioxide container.
6. Place the vessel in the incubator.

Hydrogen Ion Concentration (pH)

The growth of microorganisms is very markedly affected by pH of the medium. Most bacteria, especially pathogenic forms, grow best near the neutral point. The pH must be adjusted to the proper value before sterilization. Methods for determining the pH of solutions can be found in

TM 8-227, "Methods for Medical Laboratory Technicians."

MEDIA PREPARATION

General Considerations

No single culture medium is satisfactory for cultivating all bacteria. A basic culture medium requires sources of carbon, nitrogen, and inorganic salts. A basic medium may require the addition of other substances, such as blood, serum, amino acids, or vitamins. The basic requirements for complex energy sources may be supplied by water extracts or infusions of meat, vegetables or yeast.

Other complex energy sources include hydrolysates or digests of protein which yield amides, amino acids, peptides, peptones and proteoses.

Additional energy sources may be supplied by carbohydrates, alcohols, organic acids, glucosides or similar materials. Carbohydrates, especially glucose, are incorporated in media used for primary isolation of the pathogens. When studies of hemolysis are to be performed, carbohydrates should be excluded from blood agar. Carbohydrates are also incorporated in culture media for differentiation between bacteria on the basis of fermentation reactions.

Culture media may be liquid or solid (gelled media). A solid medium is prepared by adding agar to a liquid nutrient medium. Agar is an inert, purified extract of *Gelidium* spp. (a type of seaweed), which is not metabolized by bacteria. A liquid medium with added agar is heated to melt the agar and subsequently sterilized. At a temperature of 96°–98° C., the agar is melted and brought into solution. Upon cooling to 40° C. the agar solidifies and congeals the medium. Bacterial cells in a liquid medium are able to multiply and move with subsequent intermixing. Bacterial cells on or in an agar medium are immobilized. If few enough individual cells are deposited on or in an agar medium, each cell will multiply to form a pure visible colony.

Agar in 1.5 to 2 percent concentration is generally used in preparing solid media. Lower concentrations of agar give a semisolid medium which is ideal for performing motility checks of pure cultures.

Classification of Media

In order to select and prepare culture media for various bacteriological analyses, it is necessary to become familiar with the many and varied types of media used. For the sake of convenience, media are classified according to the purposes for which they are generally used. The various kinds of media are defined below.

BASAL MEDIA

Basal media contain ordinary peptone, peptone and meat extract, or peptone and meat infusion, with 0.5 percent sodium chloride. Agar is added (1–2 percent) when solid media are required. Such basal media are used for growing nonfastidious organisms which comprise the majority of bacteria.

ENRICHED MEDIA

These media are usually prepared by adding blood, serum, ascitic fluid, amino acids, or other complex nutrients to a basal medium for primary isolation and subculture of fastidious organism (usually pathogens). The sterile enrichment substances must be added after sterilization of the basal media.

ISOLATION MEDIA

These media are used to obtain pure growth of pathogenic microorganisms from a heavily contaminated source such as fecal specimens. To accomplish this, specific agents are added to the media to retard or prevent growth of miscellaneous contaminants present in the specimen without appreciably affecting the growth of any pathogens present. When such media are employed in the solid state, specific carbohydrates and acid-base indicators may be added to enable gross differentiation between organisms on the basis of fermentation reactions. Isolation media are subdivided into differential, selective, and inhibitory media as described in chapter 8.

BIOCHEMICAL MEDIA

Biochemical media are employed to detect the enzymic activity of bacteria in pure culture. The ability of various organisms to hydrolyze urea to ammonia and carbon dioxide, ferment various carbohydrates to acid and gas, reduce nitrates to nitrites, or produce indole from tryptophane, are all examples of enzymic activities which are useful in differentiating bacteria. Any medium which will enable the detection of these reactions or comparable reactions may be termed a biochemical medium.

MEDIA CONTAINING ANTIBIOTICS

Since various antibiotics in appropriate concentrations are inhibitory to some organisms and noninhibitory to others, they may be used in the selective isolation of certain pathogens from specimens containing contaminating microorganisms. For example, penicillin may be incorporated in Bordet-Gengou agar in a concentration of 1 unit per ml of medium. The antibiotic will restrict growth of Gram-positive organisms of the throat and thus facilitate isolation of *Hemophilus pertussis.* Penicillin and streptomycin in combination or chloramphenicol alone may be incorporated in media for isolation of certain fungi from contaminated specimens.

Sterilization Methods

Bacteriological identification requires that pure cultures of microorganisms be studied. Since microorganisms are ubiquitous, all materials used in the cultivation of bacteria must be subject to preliminary sterilization.

The fundamental principle of media sterilization is to remove or destroy all living material on or within a medium without rendering it ineffective for cultivating the desired microorganisms. The methods most commonly used involve moist or dry heat and filtration. The particular sterilization method employed is governed by the items to be sterilized and their intended use.

The usual items of glassware must be scrupulously clean. Flasks and tubes are plugged with nonabsorbent cotton to prevent entry of bacteria after sterilization. Small portions of cotton are inserted in the mouthpiece of pipettes which are subsequently wrapped in paper or placed in metal canisters. Petri dishes must be wrapped in paper or placed in canisters. All materials are wrapped to preclude contamination after sterilization. It is a good practice to cap cotton plugs of flasks with paper or foil.

Glassware prepared in this manner will remain sterile indefinitely.

AUTOCLAVE STERILIZATION

An autoclave is a large vessel or tank into which steam is introduced under pressure thus enabling temperatures above 100° C. to be reached. A comparable device used in the home is a pressure cooker.

The principle upon which the use of the autoclave is based is that microorganisms, including heat-resistant spores, are readily killed by moist heat at temperatures somewhat above that of boiling water. Water boils when the vapor pressure is equal to the pressure of the surrounding atmosphere, hence if the pressure is increased in a closed vessel, the boiling point will rise above 100° C. In a closed system the exact temperature at which water will boil is dependent upon the pressure employed. Since it is not the pressure but the temperature which is the effective lethal agent; the autoclave is equipped with a thermometer as well as a pressure gage. The exposure time should be based on observation of the temperature reading rather than the pressure gage because upon turning on the autoclave, entrapped air may cause the pressure to build up before the desired temperature is reached. The maximum temperature corresponding to any given pressure can only be obtained when the interior of the vessel is saturated with steam. In table 4–1 the temperature attained at different pressures of saturated steam is given.

Table 4–1. Pressure Temperature Relationships in the Autoclave.

Temperature in Degrees Centigrade	*Steam Pressure in Pounds*
108	5
116	10
121	15
127	20
131	25
134	30

Only culture media or solutions which are not destroyed or altered by heat should be subjected to autoclave sterilization.

In order to achieve the desired results through autoclave sterilization, the following principles must be observed:

1. The autoclave must be in proper operating condition and should be periodically evaluated.
2. The autoclave should never be overloaded.
3. Tubes should be packed loosely in baskets or racks and never placed in containers capable of trapping air.
4. Large quantities of media in single containers should be preheated to avoid undue time lag. If possible, the distribution of such media to several smaller containers is more desirable.
5. All air must be exhausted from the autoclave and the temperature reached before timing the sterilization cycle.
6. Media should never be overautoclaved or reautoclaved.
7. Flasks and tubes should never be filled to more than two-thirds capacity.
8. All media should be removed from the autoclave as quickly as possible.
9. An autoclave temperature of 121° C. (15 pounds pressure) for 15 minutes or longer is required for the sterilization of all the materials routinely used in the bacteriological laboratory.

DRY AIR STERILIZATION

Dry air sterilization is accomplished in an electrically heated, thermostatically controlled oven. Glassware containing materials not destructible by dry heat should be rendered sterile by exposure to 170° C. for 2 hours. It is important not to overload the oven. Rubber goods and plastics should not be sterilized by this method.

The dry air sterilizer is often employed for sterilizing various items of glassware such as cotton-plugged tubes, pipettes, Petri dishes, and other nonliquid materials. After autoclaving, the dry air oven may be used to dry glassware.

When natural supplements that are heat-destructible, such as blood or serum, are incorporated in culture media, they are collected aseptically and added to the sterilized and cooled medium with aseptic precautions.

FILTRATION

Since certain media or their components are destroyed or altered by exposure to heat, it is necessary to sterilize them by filtration. Materials such as some carbohydrates, serum, ascitic fluid, and urea, potassium tellurite, are thermolabile. These substances in solution are passed through a filter of fine porosity to remove all bacteria present. Examples of different types of filters available for use are listed below:

1. Berkfield filters, manufactured from infusorial earth. They can be obtained in three porosities namely, the "V" (course), "N" (normal), and "W" (fine). Only the N and W filters of this type should be used for sterilization.
2. Chamberlain filters made of unglazed porcelain in graded porosities designated L–1, L–2, L–3, etc. The filter gradations ranging from L–5 to L–7 are bacteria-proof.
3. Pyrex brand filters consisting of pulverized glass ("fritted ware") available in "UF" or ultra fine porosity for bacterial sterilization.
4. Seitz filters consisting of compressed asbestos pads of the "K" (coarse) and "EK" (germicidal) porosities.

All the above filters except the Seitz are permanently mounted in a funnel. The Seitz employs filter pads which are clamped in the base of a metal funnel. To filter sterilize a given solution, the following procedure is used:

1. The funnel stem of the filter is inserted through a rubber stopper and then securely fitted in the mouth of the filter flask.
2. Plug the side arm of the filter flask with glass wool or cotton. Sterile wrap the filter flask.
3. Sterilize the entire assembly at 20 pounds pressure for 30 minutes.
4. Attach the side arm of the filter flask to a suction pump using firm rubber tubing that is joined through a trap to the vacuum source.
5. Pour the material to be sterilized into the funnel containing the filter and turn on the vacuum source (Richards' or electrically powered pump).
6. Allow the material to be drawn through the filter and then turn off the vacuum source.
7. After permitting the internal vacuum of the filter flask to be restored to normal atmospheric pressure, disconnect the rubber tubing.
8. Aseptically remove the funnel and cover the filter flask opening by plugging with sterile cotton.
9. Dispense the sterile filtrate aseptically to appropriate sterile containers.
10. After use, immediately begin cleaning Berkfield, Chamberlain, or Pyrex brand filters. *The Berkfield* is cleaned by successive boiling in 2-percent sodium carbonate and distilled water. This is followed by rinsing with water. *Pyrex filters* are cleaned by immediately flushing with water under pressure, placing in hot hydrochloric acid, then thoroughly rinsing with distilled water. *Chamberlain or porcelain filters* are cleaned in standard chromic acid washes, flushed thoroughly with water under pressure, and rinsed with distilled water. *The Seitz filter* pads are disposable. The metal funnel parts must be washed in good detergent and thoroughly rinsed.

ISOLATION OF BACTERIA

Disease-producing bacteria usually occur in specimens in association with other bacteria, rather than in the pure state. In laboratory identification of microorganisms it is necessary that pure cultures (cultures containing only a single species) be studied.

To secure a pure culture of a given organism from a specimen or sample containing mixed flora, it is necessary to isolate a single cell from all other cells present. The cell is cultivated in such a manner that its collective progeny remain isolated.

Either one of the following methods may be used to inoculate specimens or broth cultures to agar media to obtain isolated colonies.

Streak Plate Method

This technique entails the use of agar plates previously prepared by aseptically dispensing sterile, melted medium into sterile Petri dishes in approximately 10–20 ml amounts. After solidification the agar plates are inverted and incubated at 37° C. overnight. This will serve as a sterility check in addition to drying the agar surface. To obtain well-isolated colonies from specimens, plates are streaked as shown in figure 4–4.

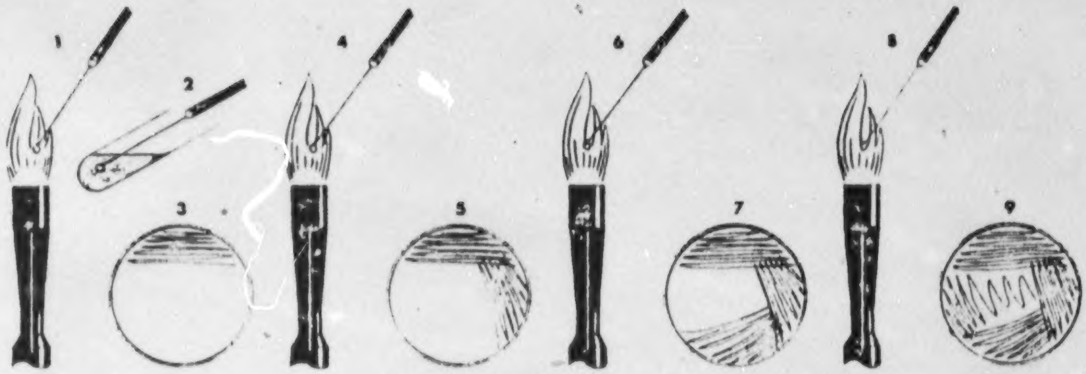

Figure 4—4. Stepwise Procedure for Streak Plate Method of Securing Isolated Colonies.

A loopful of inoculum is collected on a flame-sterilized wire loop and streaked over approximately one-quarter of the agar surface. After flaming the loop, the plate is rotated slightly and another quadrant of the agar surface is streaked. This process of diluting and spreading the inoculum over the medium is continued until the entire agar surface is covered. As the streaking continues, fewer and fewer cells remain on the loop, and finally single cells are deposited on the agar. Each isolated cell will give rise to a visible colony under suitable environmental conditions. If clinical materials on cotton swabs are to be cultured, the swab is rolled over a small area of the agar surface at the edge of the plate. With a wire loop the inoculum is spread as in figure 4–4.

The streak plate method is the most common means of securing isolated colonies, and when properly done is just as reliable and much more rapid than the pour-plate method. Although any agar base media may be used, blood agar streak plates are usually employed for primary isolation of pathogenic bacteria. After inoculation, the plates are ordinarily incubated at 37° C. for 18–24 hours in the inverted position. Growth of isolated colonies is examined grossly and microscopically for characteristics of various genera and species. Using a wire needle, pure cultures are obtained by picking growth from the center of the colony and subculturing to suitable broth or agar media.

Pour Plate Method

The pour plate technique for securing isolated colonies is generally employed using blood agar. Culture tubes containing 12 ml of sterile infusion agar, or other suitable medium are placed in boiling water to melt the agar. After cooling the medium to 48° C. in a water bath, approximately 0.7–1.0 ml of sterile, defibrinated blood is aseptically added to each tube of melted agar. Each blood medium mixture is then inoculated with a sample of the appropriate diluted specimen or broth culture, mixed well by twirling the tube and dispensed to sterile Petri dishes. It is important that the inoculum be diluted properly in preparing pour plates when many organisms are observed in the Gram smears of the specimen. This may be accomplished by inoculating a loopful of the original specimen to 5–6 ml of sterile broth or normal saline. After mixing thoroughly, one loopful of this dilution is inoculated to the melted blood agar.

Upon media solidification, individual cells in the inoculum are immobilized in various areas of the agar. During incubation each cell will multiply to form a visible colony. Blood agar pour plates are primarily used for determining the type of hemolysis produced by strains of streptococci. When subcultures or stains are to be prepared from pour plate cultures, it is necessary to secure growth from individual colonies within the agar using a sterile needle.

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Test Tube Cultures

After isolated colonies are obtained by the streak plate or pour plate technique, it is often necessary to subculture growth to tubed media to permit further study. Tubed media is prepared by dispensing broth or agar media into appropriate test tubes. The tubes are plugged with small sections of rolled cotton which are bent in the middle and inserted in the tube opening. The plug should extend about 2½ cm inside the tube and should project at least 2 cm outside the lip of the tube. Cotton plugs should fit snugly, but not so tight that difficulty will be experienced in removing and replacing the plug during bacteriological manipulations. After sterilization, broth and agar stab tubes are allowed to cool in the upright position. Agar slants are prepared by inclining the tubes of melted agar medium on a table top until solidification occurs. To prepare pure cultures, each type of medium is inoculated as follows:

1. *Liquid cultures (broth).* Using a wire loop emulsify a small amount of growth on the moist wall of the tube just above the liquid and wash down by tilting the tube. If the inoculum is liquid, a loopful is simply placed in the broth and dispersed by gentle agitation.
2. *Slant cultures.* Slant cultures are prepared by streaking the inoculum over the slant surface from bottom to top. If the slant contains some water of condensation at its base, do not spread the water over the surface of the slant since the resulting growth will not yield characteristic colony appearance.
3. *Stab cultures.* Stab cultures are made with a straight needle into tubes of unslanted solid or semisolid medium. The stab line, centered without lateral movement, should extend approximately one-half to two-thirds the depth of the medium.

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Chapter 17

ORGANIZATION AND MANAGEMENT OF A BACTERIOLOGY LABORATORY

Bacteriology laboratories should be isolated from other departments in the clinical laboratory, especially those having direct patient relationship (blood collecting room, blood bank, etc.). Traffic should be kept to a minimum and patients should not be allowed to enter the bacteriology laboratory.

Rooms should have adequate ventilation and heating systems. The heating system should maintain stable humidity and temperature, which are important factors for maintaining the proper function of incubators.

Adequate lighting is essential for all laboratory work. Whenever possible, the bacteriology laboratory should have window exposure to daylight. Daylight type flourescent lighting is recommended when artificial lighting is required. Improper lighting causes fatigue, eyestrain and errors in interpreting bacteriologic reactions.

Rooms should be free of dust, for dust may contain contaminating organisms. Precautions that may be taken to control dust are:

1. Change heating and air conditioning system filters, frequently.
2. Install gauze filters for nonfiltered heating and ventilating ducts.
3. Oil working surfaces of benches and cabinets.
4. Sweep with moist sawdust or sweeping compound.
5. Damp mop the floors before pouring media.

Preventive maintenance on equipment should be practiced. Incubators, centrifuges, water baths, sterilizers, microscopes and lamps should be checked frequently for operational efficiency. Daily checks should be made on incubators and water baths to insure proper temperature and water level. The use of a checklist is helpful in accomplishing this task.

HANDLING INFECTIOUS MATERIAL

Personnel should wear laboratory coats or smocks while working in the laboratory. Hands will be washed thoroughly with surgical soap or nonirritating antiseptic after handling cultures and specimens. Care should be taken to protect the eyes, abrasions, or cuts from microorganisms; any accidental infection should be reported to a medical officer for advice and prophylactic treatment.

Specimens should be carefully handled and examined to prevent laboratory infection. Paper toweling may be wrapped around containers to avoid direct contact with possible contaminated surfaces. When practical, use leakproof, screw-capped containers for obtaining specimens and holding specimens during analyses.

Immediately after use, contaminated materials such as specimen containers, swabs, pipettes, or instruments should be autoclaved or placed in receptacles containing disinfectant solution. When analyses have been completed, specimens should be sterilized in the autoclave and appropriately discarded.

All cultures should be autoclaved after identification is obtained. A good practice is to autoclave such cultures, with cotton plugs and lids removed, in a pail of water containing glassware detergent. This treatment will destroy all bacteria and permit easy cleaning of glassware.

The working surfaces of the laboratory should be swabbed with phenol, cresol, or other effective antiseptic before and after working with infectious materials. It is a wise practice to work over cloth or paper towels soaked in disinfectant to prevent contamination of laboratory benches. This is an absolute necessity when dealing with specimens containing highly invasive pathogens such as *Brucella* spp. and *Mycobacterium tuberculosis.*

Personnel assigned to work in the bacteriological laboratory should be carefully indoctrinated in the sterile techniques required. A thorough "check-out" in the proper use of the bacteriologic needle in inoculating cultures is mandatory. Work with highly infective and dangerous organisms should only be attempted by a technician well trained in bacteriology and having full knowledge regarding the materials and equipment at his disposal. Special equipment, e.g., a bacteriological hood, may be imperative before embarking on certain bacteriological studies.

CARE OF GLASSWARE

New glassware may contain free alkali and should be rinsed thoroughly in hot tap water, then several times with distilled water prior to use.

New microscope slides and cover slips should be placed in 95 percent ethyl alcohol and dried prior to use. Microslides and cover slips should be discarded after using. Artifacts from stains and scratches make re-use impractical.

Contaminated glassware should be autoclaved (chapter 4), then washed in hot tap water containing glassware detergent. Tubes, flasks, and beakers will usually need a thorough scrubbing with a brush. After scrubbing, rinse all glassware in tap water, then several times with distilled water.

Clean pipettes by soaking them in a cylinder containing water and detergent. Rinse the pipettes with running tap water and allow to drain. The pipettes are placed overnight in an acid cleaning solution. Rinse the pipettes with running tap water. Place the pipettes in a pipette washer for several hours. Rinse the pipettes several times in distilled water.

Dry the glassware by draining it upside down or by placing it in a hot air oven. The dry glassware may be stored or prepared for sterilization (chapter 4).

PROCUREMENT OF SUPPLIES AND EQUIPMENT

The request for standard, stocked supplies and equipment is initiated by preparing DD Form 1150 (Request for Issue or Turn-In), in six copies and submitting five of the copies to the medical supply officer. Stock numbers and item descriptions may be found in the Federal Standard Stock Catalogue.

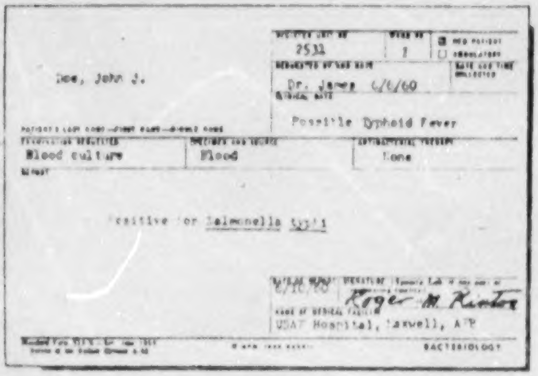
Non standard supplies and equipment must be requisitioned locally from a commercial source. These requests will be handled by the medical supply officer.

Certain non-standard biologicals may be requisitioned by letter from the Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington 12, D.C.

RECORDS AND REPORTS

Requests for bacteriological analyses must be submitted on Standard Form 514K, Bacteriology (fig. 17-1).

In addition, requests must include source of specimen, type of examination requested, and anti-bacterial therapy administered. A separate request slip is required for each type of examination, e.g., culture, AFB concentration, fungus examination, antibiotic sensitivity test, etc. Reports of laboratory findings will be distributed to requesting facilities on the original copy. The carbon copy will be retained in the laboratory file.



Doe, John J.

2531

1

Dr. James 6/6/60

Possible Typhoid Fever

Blood culture

Blood

None

Positive for Salmonella typhi

6/10/60

Roger M.

USAF Hospital, Maxwell, AFB

BACTERIOLOGY

Figure 17-1. Standard Form 514-K, Bacteriology.

A permanent laboratory worksheet, or log must be maintained on all work performed. This log must contain the date, patient's name, ward number, requesting physician, type of specimen, type of examination, results and the number assigned to the specimen. The worksheet or log must be kept in the laboratory as a permanent record. There are many satisfactory systems for numbering the specimens. It is suggested that specimens processed be numbered chronologically each month. This will aid in enumerating the total completed test procedures for monthly reports.

CARE OF LABORATORY ANIMALS

Animal rooms must be kept clean, dry, warm and free of insects and rodents. At least twice a week, cages must be cleaned and the sawdust for droppings changed.

A satisfactory diet for most laboratory animals (mice, rats, rabbits) is supplied by commercial, dry feed. There must be fresh, clean water available to the animals at all times.

To determine if new animals are free from disease, isolate the new animals from the regular stock for at least ten days. Dead animals should be wrapped in paper and immediately incinerated.